Paediatric asthma: from environmental determinants towards diagnostic breathomics

Asma em idade pediátrica: dos determinantes ambientais ao diagnóstico por perfis de ar exalado

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List of publications

The present thesis is based on the following publications, which will be referred in the manuscript by their roman numerals **I - IV**:

I. Cavaleiro Rufo, J., Madureira, J., Paciência, I., Aguiar, L., Pereira, C., Silva, D., Padrão, P., Moreira, P., Delgado, L., Annesi-Maesano, I., Oliveira Fernandes, E., Teixeira, J. P. & Moreira, A. 2017. Indoor fungal diversity in primary schools may differently influence allergic sensitization and asthma in children. Pediatr Allergy Immunol, 16, 12704.

II. Cavaleiro Rufo, J., Paciência, I., Silva, D., Martins, C., Madureira, J., Oliveira Fernandes, E., Padrão, P., Moreira, P., Delgado, L. & Moreira, A. 2018. Swimming pool exposure is associated with autonomic changes and increased airway reactivity to a beta-2 agonist in school aged children: A cross-sectional survey. PLoS ONE, 13(3): e0193848.

III. Cavaleiro Rufo, J., Madureira, J., Oliveira Fernandes, E. & Moreira, A. 2016. Volatile organic compounds in asthma diagnosis: a systematic review and meta-analysis. Allergy, 71, 175-88.

IV. Cavaleiro Rufo, J., Paciência, I., Castro-Mendes, F., Farraia, M., Rodolfo, A., Silva, D., Oliveira Fernandes, Delgado, L. & Moreira, A. 2018. Exhaled breath condensate volatilome allows sensitive diagnosis of persistent asthma. (*Submitted to the Allergy journal. Accepted with revisions at the time of printing*).

Abstract

Asthma is the leading non-communicable disease among children. Although several environmental determinants have already been associated with the development of asthma, more research is needed to understand how to manage the exposure to these determinants in order to prevent the disease onset. Moreover, the diagnosis and phenotyping of asthma is particularly complex in children due to concomitant virus-induced symptoms, transient wheezing and the difficulty in obtaining history of recurrent symptoms or exacerbations, which may lead to misdiagnosis and inappropriate treatment. Therefore, this thesis aimed to provide further insights on the development and diagnosis of paediatric asthma in order to improve its prevention and treatment.

This thesis is based on four studies: 1) a cross-sectional analysis of schoolchildren exposed to different microbial diversities in classrooms; 2) a cross-sectional survey between schoolchildren with different degrees of exposure to indoor swimming pool environments; 3) a systematic review and meta-analysis of studies using exhaled volatile organic compound measurements for asthma diagnosis; and 4) a cross-sectional study to distinguish individuals with paediatric asthma trough electronic nose analysis of volatile organic compounds in exhaled breath condensate samples.

Classrooms with increased diversity scores showed a significantly lower prevalence of children with allergic sensitization, but presented a tendency for a higher risk of functional asthma. The risk of allergic sensitization increased with increasing endotoxin and *Penicillium spp* exposure in classrooms.

On the other hand, children who swam had significantly lower maximum and average pupil constriction velocities when compared to both past swimmers and non-swimmers (3.8 and 5.1 vs 3.9 and 5.3 vs 4.0 and 5.4 mm/s, respectively). Moreover, levels of exhaled nitric oxide and affinity to the beta-2 agonist were significantly higher in current swimmers when compared to non-swimmers (70 and 12 vs 60 and 10, respectively in mL and ppb).

The systematic review and meta-analysis showed that: firstly, exhaled volatile organic compound profiles have high sensitivity and specificity values for asthma diagnosis; secondly, individuals with asthma had 6 times higher chance of being diagnosed through analysis of exhaled volatile organic compounds than healthy controls; and thirdly, diagnosis odds ratios and AUC values were 49.3 and 0.94, respectively.

The diagnostic accuracy measurements for our exhaled volatile organic compound analysis methodology showed AUC values of 0.81 for both asthma diagnosis and persistent asthma diagnosis. Although specificity values were generally lower, accuracy, sensitivity and AUC parameter values obtained from exhaled volatile organic compound analysis surpassed those from spirometry with bronchodilation in all cases.

Altogether, this thesis showed that exposure to microbial agents in classrooms and disinfection by-products in swimming pools does influence the development or exacerbation of asthma in school-aged children. Moreover, the demonstrated methodology for exhaled volatile organic compound analysis may not only corroborate paediatric asthma diagnosis, but may also assist physicians in their decision to administer inhaled corticosteroids. Through these developments, the present thesis is expected to contribute to the improvement of both prevention and treatment of paediatric asthma.

Resumo

A asma é a doença não-transmissível mais comum entre crianças. Embora vários determinantes ambientais tenham já sido associados ao desenvolvimento de asma, é necessária uma investigação mais aprofundada para compreender como podemos manipular a exposição a estes determinantes, de forma a prevenir o aparecimento da doença. É também necessário ter em conta a complexidade do diagnóstico e fenotipagem da asma em idade pediátrica, muito por causa da semelhança dos sintomas associados a doenças concomitantes, igualmente comuns neste grupo etário, como sintomas induzidos por vírus ou pela pieira intermitente, e que, quando associados à dificuldade em obter o histórico clínico, podem levar a um diagnóstico errado e, consequentemente, a um tratamento inapropriado. Tendo isto em conta, procurou-se esclarecer hipóteses sobre o desenvolvimento e diagnóstico da asma pediátrica, de forma a melhorar a sua prevenção e tratamento.

Esta tese tem por base quatro estudos: 1) uma análise transversal de crianças em idade escolar expostas a diferentes níveis de diversidade microbiológica presentes nas salas de aula; 2) um estudo transversal entre crianças com diferentes níveis de exposição ao ambiente em piscinas interiores; 3) uma revisão sistemática com meta-análise de estudos sobre medição de compostos orgânicos voláteis no ar exalado para diagnóstico de asma; e 4) um estudo transversal focado em distinguir indivíduos com asma em idade pediátrica, utilizando um método baseado num sistema de nariz eletrónico para análise dos compostos orgânicos voláteis em amostras de condensado de ar exalado.

As salas de aula que apresentaram uma maior diversidade fúngica estavam associadas a uma menor prevalência de sensibilização alérgica, mas mostraram uma tendência para um maior risco de asma com alteração da função pulmonar. O risco de sensibilização alérgica aumentou com uma maior exposição a endotoxinas e *Penicillium spp* nas salas de aula.

Por outro lado, crianças que praticam natação mostraram uma diminuição significativa da velocidade média e máxima de constrição da pupila, quando comparadas com anteriores praticantes de natação, ou com as que nunca praticaram natação (3.8 e 5.1 vs 3.9 e 5.3 vs 4.0 e 5.4 mm/s, respetivamente). Para além disso, os níveis de óxido nítrico exalado e a afinidade ao agonista beta-2 estavam significativamente elevados nos praticantes de

natação, quando comparados com os que nunca praticaram (70 e 12 vs 60 e 10, respetivamente em mL e ppb).

A revisão sistemática com meta-análise demonstrou que: primeiro, os perfis de compostos orgânicos voláteis no ar exalado apresentam alta sensitividade e especificidade para o diagnóstico de asma; segundo, indivíduos com asma têm 6 vezes mais probabilidades de serem diferenciados do que os saudáveis; e terceiro, a rácio de diagnóstico assertivo e os valores de AUC são respetivamente 49.3 e 0.94.

A nossa metodologia de análise dos compostos orgânicos voláteis no ar exalado apresentou valores de AUC de 0.81, tanto para o diagnóstico de asma em idade pediátrica, como para a diferenciação de asma persistente. Apesar de menos específica, a análise dos compostos orgânicos voláteis no ar exalado apresentou valores de precisão, sensitividade e AUC superiores aos da espirometria com prova de broncodilatação em todos os casos.

Em síntese, esta tese comprovou que a exposição a agentes microbiológicos em salas de aula e a produtos de desinfeção em piscinas interiores influencia tanto o desenvolvimento de asma, como a sua exacerbação, nas crianças em idade escolar. Para além disso, a metodologia apresentada para a análise dos compostos orgânicos voláteis no ar exalado pode não só corroborar o diagnóstico de asma em idade pediátrica, mas também auxiliar os médicos na decisão de administrar terapia por corticoide inalado. Através destes desenvolvimentos, a presente tese poderá contribuir para a melhoria da prevenção e tratamento da asma em idade pediátrica.

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Acronyms

ACV	Average constriction velocity
ADV	Average dilation velocity
ANOVA	Analysis of variance
ATS	American Thoracic Society
AUC	Area under the ROC curve
BAL	Bronchoalveolar lavage
BMI	Body mass index
CFU	Colony-forming unit
CI	Confidence interval
CON	percentage of pupil constriction
COPD	Chronic obstructive pulmonary disease
CS	Current swimmers
DALYs	Disability adjusted life years
DOR	Diagnostic odds ratio
EBC	Exhaled breath condensate
eNose	Electronic nose
EU	Endotoxin unit
FEF25-75	Forced expiratory flow 25-75%
FEV1	Forced expiratory volume at the first second
FVC	Forced vital capacity
GC	Gas-chromatography
GC-DMS-MS	GC-MS with differential mobility spectrometry
GC-FID	Gas chromatography coupled with flame ionization detector
GC-MS	Gas-chromatography coupled to mass-spectrometry
GCxGC-ToF-MS	Double GC coupled to a time-of-flight mass-spectrometer
GORD	Gastro-oesophageal reflux disease
IgE	Immunoglobulin E
IMS	Ion mobility spectrometry
IOS	Impulse oscillometry
ISAAC	International study of asthma and allergies in childhood
LPS	Lipopolysaccharide (Gram-negative endotoxin)

MCV	Maximum conscription velocity
NIOSH	National Institute for Occupational Safety and Health
NLR	Negative likelihood ratio
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NS	Non-swimmers
OR	Odds ratio
PCA	Principal component analysis
PEF	Peak expiratory flow
PLR	Positive likelihood ratio
PRISMA	Preferred reporting items for systematic reviews and meta-analysis
PS	Past swimmers
QUADAS	Quality assessment of studies of diagnostic accuracy included in systematic reviews
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SD	Standard deviation
SPT	Skin-prick-tests
STARD	List of essential items for reporting diagnostic accuracy studies
T75	Time in seconds at 75% recovery of pupil size
TRPV1	Transient receptor potential vanilloid 1
VOC/VOCs	Volatile organic compound/s

1 Introduction

Asthma is the leading non-communicable disease among children, with prevalence reaching up to 10% of the paediatric population in most industrialized countries (Braman, 2006, Asher and Pearce, 2014). Some epigenetics and environmental determinants behind the development of paediatric asthma have already been revealed by the scientific community, but further research is needed to completely understand the triggers behind its onset (Burbank *et al*., 2017, Yang *et al*., 2017).

Indoor air represents one of the most relevant means of environmental exposure during childhood, and indoor air microbiological agents have been shown to influence the development of allergic diseases (Karvonen *et al*., 2014). Apart from home, young children spend a large section of their daytime at school, mostly within their classrooms, which reflects a long term exposure to indoor air microbiota (Ege *et al*., 2011, Morawska *et al*., 2013). If the human immune system does change according to environmental exposure in the first years of life, the classroom microbial environment might contribute to either the development and/or exacerbation of asthma or, on the other hand, have a protective effect against its onset. However, home and classrooms are not the only environments of significant exposure in the paediatric population.

Notwithstanding the recognized health benefits associated with the practice, swimming pool attendance is known to be responsible for airway dysfunction in swimmers due to chlorine-based disinfection by-products exposure (Bernard et al., 2007, Bernard et al., 2015). These products are ingested, inhaled or absorbed via skin during the swimming practice, possibly resulting in airways epithelium damage and eventually airway hyperresponsiveness (Jacobs *et al*., 2007, Belda *et al*., 2008). This has been emphasized by several studies published in the last decade, showing an association between swimming pool attendance and a higher risk of asthma in children (Bernard et al., 2009, Voisin et al., 2010, Andersson et al., 2015), which has lead the scientific community to question the beliefs regarding swimming practice benefits in the paediatric population (Uyan *et al*., 2009). Alterations of the autonomic nervous function have also been associated with swimming pool attendance, although endurance exercises and extensive training volume are frequently held responsible for these changes somehow disregarding the possible influence of the indoor swimming pool environment on the parasympathetic tonus. It is therefore possible that environmental exposure in swimming pools may be causing

dysautonomia in swimmers, independently of the training volume, ultimately leading to development of asthma, even in children swimmers.

Aside from environmental determinants, asthma diagnosis is an intricate challenge in the paediatric population. The diagnosis and phenotyping of asthma is particularly complex in children due to concomitant virus-induced symptoms, transient wheezing and the difficulty in obtaining history on recurrent symptoms or exacerbations, which may lead to misdiagnosis and inappropriate treatment (Contoli and Papi, 2010, Baena-Cagnani and Badellino, 2011, Lodrup Carlsen et al., 2011, Konradsen et al., 2015). Therefore, a more sensitive biomarker to diagnose asthma in children, combining versatility, non-invasiveness and accuracy, is needed.

In the last decade, exhaled volatile organic compounds (VOCs) have been increasingly used as biomarkers for several diseases, including asthma (Dallinga et al., 2010, Ibrahim et al., 2011, Caldeira et al., 2012, Smolinska et al., 2014). Several research groups were able to analyse exhaled VOCs, creating models or profiles capable of accurately distinguishing individuals with asthma. These biomarkers showed high sensitivity and overall accuracy for detecting asthma inflammation, which could be proven useful in a real clinical context. Nevertheless, most of these studies lack stability measurements and external validation methods. Moreover, almost all of the studies used exhaled breath as the VOC collection matrix, which has some inherent limitations that would complicate the implementation of exhaled VOC analysis in clinical settings. Studies have shown that it is also possible to measure VOCs in exhaled breath condensate (EBC), which would be a more convenient matrix for storage, transport and analysis. There are, however, no published studies concerning exhaled VOC analysis measured in EBC samples for asthma diagnosis or severity monitoring.

Therefore, this thesis aimed to provide further insights on the development and diagnosis of paediatric asthma.

2 Literature review

2.1 Paediatric asthma

2.1.1 Definition and epidemiology

Asthma is a complex chronic respiratory disease characterized by airflow obstruction, bronchial inflammation and hyperresponsiveness, multiple phenotypes and endotypes, with several aetiologies for its onset and exacerbation of symptoms (Lemanske and Busse, 2010). These symptoms include recurrent episodes of wheezing, dyspnoea, chest tightness, and dry cough, particularly at night or in the early morning, and the overall inflammation process involves activation of mast cells, eosinophils, T lymphocytes, neutrophils, and epithelial cells, with a tendency for a Th2-mediated inflammation profile in atopic individuals. Airflow obstruction in asthma can be totally, partially, or not reversible without proper medication, and may be resultant from multiple structural and/or physiologic factors associated with the disease that individually or collectively contribute to airway narrowing, such as airway smooth muscle spasm, airway mucosal oedema, mucus hypersecretion, and airway remodelling and inflammation (Lemanske and Busse, 2003). For many asthmatic patients, the disease usually develops in early childhood. Although wheezing episodes may occur during the first three years of life in approximately 50% of the paediatric population, some of these children will stop wheezing (transient wheezers), while others will go on to develop persistent symptoms that will either dissipate prior to adolescence, or continue into adulthood (Stern *et al*., 2008). Moreover, although some phenotypes of asthma require less effort to control, some children may develop a more severe, generally more Th1-medited, asthma phenotype, which requires continuous or near continuous treatment through a combination of oral and inhaled corticosteroids with a short-acting β-agonist to achieve a controlled state (Wenzel and Busse, 2007).

Asthma is the leading non-communicable disease among children and is prevalent in approximately one tenth of the paediatric population in most industrialized countries (Braman, 2006). According to the Global Asthma Report (2014) and the Institute for Health Metrics and Evaluation, asthma in individuals aged from 5 to 19 years old results in a burden of approximately 300 to 600 disability adjusted life years (DALYs), the highest after the elderly population. When children with asthma experience exacerbations, they are often unable to carry out their usual educational activities and may need urgent medical

treatment, leading to high hospital admissions. In extreme cases, children may actually die from severe symptoms of asthma (Asher and Pearce, 2014). These complications result in high associated health care costs, namely in reliever medication, emergency and monitoring instrumentation, ambulatory procedures, hospital care, asthma management planning and patient education, not accounting the productivity losses associated with absenteeism (Bahadori *et al*., 2009). In Portugal, the average annual cost of a single child with asthma exceeds the 600 euros, which represents a total expense of 123 million euros per year, considering an estimated asthma prevalence of 11.5% (van den Akker-van Marle *et al*., 2005, EUROSTAT, 2014).

There are several known determinants of asthma inception and exacerbation. Although genetic factors have been shown to contribute significantly to disease expression and severity, asthma is genetically classified as a complex disorder and does not follow simple Mendelian inheritance characteristics (Ober and Hoffjan, 2006, Lemanske and Busse, 2010). Therefore, non-genetic risk factors must be taken into consideration and some of the most important determinants in paediatric asthma are prompted through environmental exposures.

2.1.2 Environmental determinants

Environmental exposure plays an important role in the onset and exacerbation of paediatric asthma. Aeroallergens, environmental pollution, tobacco smoke and biodiversity, all have been shown to partake as environmental determinants (Lemanske and Busse, 2010).

As shown in a study by Arbes *et al.* (2007) in the United States, where 56.3% of asthma cases were attributable to atopy, exposure to aeroallergens is one of the most important factors for onset and, predominantly, exacerbation of the disease. Since the formation of antigen-specific IgE antibodies to aeroallergens does not usually occur until 2 to 3 years of life, aeroallergen-induced asthma is uncommon during early childhood but begins to increase in prevalence during subsequent years, peaking in the second decade of life (Murray et al., 2001, Lemanske and Busse, 2003). When a sensitized individual is exposed to specific inhaled allergens, acute asthma symptoms may occur in combination with exacerbated airway inflammation. The mechanisms connecting these allergens to the development of asthma are not fully established but the significant evidence associating

them with asthma morbidity assures their position as environmental determinants (Ahluwalia and Matsui, 2011).

Early-life exposure to tobacco smoke continues to be a significant determinant in asthma. Studies have shown that exposure to tobacco smoke increases the expression of arginase I in individuals with asthma, altering arginine pathways in the airways and increasing airway remodelling (Bergeron *et al*., 2007). Added effects from tobacco smoke exposure have also been associated with a higher risk of polymorphisms in beta2-adrenergic receptor gene during the gestational period, increasing the risk for childhood wheezing and, eventually, asthma (Wang *et al*., 2008).

However, tobacco smoke is not the only air pollutant to be associated with asthma. Since children spend most of their time indoors, particular attention should be given to indoor air quality to further understand the environmental determinants in paediatric asthma. Studies have shown that prolonged exposure to coarse, fine and ultrafine particulate matter in indoor environments may promote the development of respiratory diseases (MacNee and Donaldson, 2003, Cavaleiro Rufo *et al*., 2016a). Irritant and toxic effects have also been observed for many exogenous VOCs, mostly prevenient from every-day products such as cleaning detergents (Chin et al., 2014, Cavaleiro Rufo et al., 2016b, Paciencia et al., 2016). Nevertheless, Nurmatov *et al.* reviewed, in 2015, a series of studies regarding indoor air VOC exposure as determinants of asthma and concluded that "the available evidence implicating domestic VOC exposure in the risk of developing and/or exacerbating asthma and allergy is of poor quality and inconsistent", thus further research needs to be commenced to shared more light on this subject. Airborne microbial agents have also been suggested as a risk factor for asthma, promoting airway inflammation and increasing oxidative stress (Mendell *et al*., 2011, Madureira *et al*., 2015). However, these results need to be interpreted with caution, since the recent biodiversity hypothesis considers a balanced microbial exposure to be a preventive factor in asthma development (Haahtela, 2014). Shortly, early contact with multiple microbiological agents appears to contribute to a preventive immunomodulation, inducing tolerance to allergens through both adaptive and innate immune mechanisms, ultimately reducing the risk for allergic diseases and asthma in later childhood (Cavaleiro Rufo *et al*., 2017b).

Nevertheless, either as a promoter or preventer, environmental exposure is an important determinant for paediatric asthma and therefore needs to be further studied. Unfortunately, most of the published studies only focus on home exposure and, although

children do spend most of their time at home, there are other important spaces where considerable environmental exposure occurs, such as classrooms, gymnasiums or indoor swimming pools.

2.1.3 Diagnostic challenges

Asthma diagnosis in the paediatric population is usually more complicated since there are currently no implemented clinical tools to discriminate true asthma from isolated symptoms, such as episodic wheezing (Neerincx *et al*., 2017). As previously mentioned, almost 50% of children present any form of wheezing episodes at 6 years of age that may be triggered by several conditions not associated with asthma, including viral respiratory tract infections, cystic fibrosis, malformations of the respiratory system, foreign body aspiration, and gastroesophageal reflux, among others (Lemanske and Busse, 2003). Eventually, diseases with concomitant symptoms can hind or mask asthma diagnosis, and may even lead to over-diagnosis in some cases. In addition, the existing clinical biomarkers are incapable of properly identifying different phenotypes of asthma, thus resulting in unoptimized treatment even when an accurate confirmation of asthma has been achieved (Chung, 2016).

Currently, and according to the Global Initiative for Asthma, clinical approaches for asthma diagnosis include the analysis of medical history (occurrence of symptoms, allergen sensitization and exercise-induced bronchoconstriction), physical examination (wheezing auscultation and inspection for physical signs of airflow limitation), and diagnostic tests, such as lung function testing, fractional exhaled nitric oxide (NO) measurements, bronchoprovocation (i.e. methacholine or histamine), skin-prick-tests (SPT) and sputum or bronchoalveolar lavage (BAL) eosinophils count (Global Initiative for Asthma, 2017). However, these methods have multiple downsides, either considering accuracy, invasiveness, and/or expensiveness. In addition, some of these tests require cooperation from the patient, which could result in poorly reproducible results when dealing with younger elements of the paediatric population. Table 1 summarises the strengths and limitations of the most frequently used clinical tools for asthma diagnosis.

Table 1 – Summary of the currently adopted clinical tests for asthma diagnosis and phenotyping.

Method	Assessed parameters	Strengths	Limitations
Spirometry with bronchodilation challenge	- Lung function - Airway obstruction and reversibility	- High specificity and reproducibility - Highly standardized	- Low sensitivity - Effort-dependent - Less reliable in children
Bronchoprovocation challenge tests	- Airway responsiveness	- High sensitivity	- Low specificity - Bronchoprovocation may cause discomfort
Impulse oscillometry with bronchodilation challenge	- Lung function - Airway obstruction and reversibility	- Effort-independent - Able to identify proximal or distal airway obstructions - Appropriate for young children and frail elderly	- Lacks proper standardization - Interpretation of results is not as straightforward as spirometry
Exhaled nitric oxide measurements	- Airway eosinophilic inflammation	- Fast and easy to perform - May assist in asthma severity monitoring - Decent indicator for allergic asthma	- Requires complementary diagnostic approaches - Levels are usually high in atopic individuals regardless of having asthma or not
Skin-prick-tests	- Allergic sensitization	- High sensitivity - Cost-effective - Rapid to perform	- Positive response does not necessarily mean allergic disease
Sputum eosinophils count	- Eosinophilic inflammation	- Direct marker - Decent accuracy	- Requires laboratory analysis - Sputum induction causes discomfort to patient - Less useful for neutrophilic asthma phenotypes

Spirometry is the standard method for measuring airway obstruction and associated reversibility, which are major indicators of asthma pathophysiology. Shortly, measurements of forced expiratory volume at the first second (FEV_1) and forced vital capacity (FVC) are conducted during forced expiratory manoeuvres, using a spirometer. A bronchodilation challenge may be conducted between two spirometry measurements, with an increase of at least 12% and 200mL in FEV_1 from baseline to post-bronchodilator assessment being accepted as a positive diagnosis for asthma (Global Initiative for Asthma, 2017). Although spirometry with bronchodilation challenge provides highly specific and reproducible results, it has an unpractically low sensitivity for paediatric asthma diagnosis.

In fact, Schneider *et al.* (2009) performed a study with the spirometry assessment of 219 patients with obstructive airways diseases, and concluded that asthma could not be excluded using only spirometry. These results were later supported by Tse and co-workers (2013), who tested this method on 1041 children with mild to moderate asthma, reporting a sensitivity of only 35.6%, despite obtaining an area under the ROC (AUC) of 0.73. In addition, spirometry is effort-dependent and the prediction values are less reliable in young individuals, thus not always being the optimal approach for paediatric asthma diagnosis (Global Initiative for Asthma, 2017).

Bronchoprovocation challenge may be performed to measure airway responsiveness in patients with symptoms consistent with asthma but showing normal lung function. Measurements of airway responsiveness to direct airway challenges, such as inhaled methacholine and histamine, or indirect airway challenges, such as inhaled mannitol or exercise challenge, may help in asthma diagnosis and phenotyping (Anderson, 2008, Global Initiative for Asthma, 2017). Generally, test results are usually expressed as the provocative concentration of the agonist causing a given fall of at least 20% in FEV_1. However, although bronchoprovocation challenge tests are more sensitive than spirometry for asthma diagnosis, they are short on specificity, thus generating a significant amount of false positive results (Cockcroft, 2010, Kim *et al.*, 2014). Moreover, provoking bronchospasm may cause considerable discomfort in patients, particularly in children.

Impulse oscillometry (IOS) is another method to evaluate lung function and has also been used to measure bronchodilator response and bronchoprovocation testing. It is performed during normal tidal breathing and, unlike spirometry, requires no effort from the patient, which is an important asset in paediatric asthma diagnosis (Manoharan *et al.*, 2015). However, studies have shown that, although capable of detecting proximal or distal airway obstructions, IOS still lacks proper standardization and thus interpretation of its results are not as straightforward to the practitioner as spirometry. Therefore, IOS is mainly used in predicting loss of asthma control in the paediatric and elderly populations (Bickel *et al.*, 2014).

Exhaled nitric oxide is a biomarker of eosinophilic inflammation and has been suggested as a convenient indicator of airway inflammation in asthma due to its non-invasiveness, rapid testing, and ease of use (Smith *et al.*, 2004). Hence, exhaled NO is frequently used in clinical studies to identify individuals with more severe phenotypes of asthma, which are normally associated with a higher hyperresponsiveness (Gemicioglu *et*

al., 2014). Our group has recently suggested and published a spirometry adjusted exhaled NO index, which showed a good performance in assessing asthma control in children (Martins *et al*., 2017). Nevertheless, exhaled NO measurement is not an useful tool for paediatric asthma diagnosis since levels are generally increased in atopic children regardless of whether they have asthma or not, and should therefore be seen as a complementary tool for asthma phenotyping (Prasad *et al*., 2006).

Due to the strong association between asthma and allergic rhinitis, determining the allergic status of the patient is usually helpful to identify the phenotype of the disease. Skin-prick-tests with allergens are often used as the primary diagnostic tool in determining allergic status, since they are cost-effective, have high sensitivities and can be rapidly performed (Bernstein *et al*., 2008). Allergic sensitization is usually defined by a positive SPT to at least one of the tested allergens (wheal > 3mm) coupled to a positive histamine response (wheal > 3mm) and no positivity in the negative control (wheal < 3mm). However, these tests cannot be used to diagnose asthma on their own, and a positive response does not necessarily mean that the patient suffers from allergic disease or from a Th2-mediated allergic asthma (Global Initiative for Asthma, 2017).

Eosinophilic inflammation can be directly measured through sputum eosinophils count, providing valuable information regarding the cellular and molecular processes in eosinophilic-mediated asthma (Romagnoli *et al*., 2002, Spahn, 2012). This technique presents several limitations, however, as sputum induction provides some discomfort to the patient (which is less tolerable in children), requires laboratory analysis, and may not generate good diagnostic results for the more neutrophilic-mediated asthma phenotypes (Covar *et al*., 2004).

Table 2 summarizes the sensitivity and specificity values of currently used methodologies to assist in asthma diagnosis. Other invasive techniques, such as bronchoscopy and biopsy, are highly accurate for examining airway inflammation, but are rarely used due to their associated risks.

Table 2 - Sensitivity and specificity for each diagnostic test for asthma. Adapted from Rufo *et al.* (2016b) with authorization from the copyright holder.

Diagnosis Method	Reference	Asthma patients (n)	Sensitivity (%)	Specificity (%)
Spirometry	(Schneider *et al*., 2009)	90	16	100
Fev1 < 80% predicted	(Smith *et al*., 2004)	17	29	100
Fev1 < 90% predicted	(Smith *et al*., 2004)	17	35	93
Bronchodilator reversibility > 12%	(Tse *et al*., 2013)	1041	36	90
Sputum Eosinophils	(Smith *et al*., 2004)	14	86	88
Exhaled NO > 20 ppb	(Smith *et al*., 2004)	16	88	79
Exhaled NO >46 ppb	(Schneider *et al*., 2009)	83	32	93
GINA questionnaire ≥ 1 symptoms	(Lim *et al*., 2014)	164	98	9
GINA questionnaire ≥ 5 symptoms	(Lim *et al*., 2014)	164	19	92

2.1.4 Future implications

Paediatric asthma is a troublesome disease affecting a large number of children, generating large annual expenses associated with both hospital care and treatment. Cultivating preventive actions and enhancing diagnostic approaches would not only reduce the burden of disease and associated costs, but also improve treatment administration through more specific medication. However, there is still much to unravel regarding mechanisms of paediatric asthma inception, particularly concerning environmental exposure in children. Moreover, improved methods for asthma diagnosis are required, as the evident limitations of the diagnostic tools currently used in clinical settings poorly assist physicians in administrating the correct treatment.

Concerning environmental exposure, special attention should be given to public environments, such as schools or other learning facilities, since children spend a large part of their time in these environments, corresponding to significant periods of exposure that may determine the onset of paediatric asthma (Morawska *et al*., 2013). On the other hand, prediction of therapy response would benefit from non-invasive and easily accessible biomarkers of paediatric asthma, preferably if effortlessly gatherable in children at point of care. Therefore, breathomics technologies are starting to appear as appealing tools for asthma diagnosis among the scientific and medical communities (Boots *et al*., 2015).

2.2 Breathomics in asthma

Citing Bos *et al.* (2016), "among the various omics technologies, those that can be measured at point of care are likely to prevail in clinical practice". This is the reason why the practical, rapid and non-invasive metabolomic analysis of exhaled air (breathomics) is becoming such an appealing option in modern medicine.

The perception of different metabolites released through the exhaled breath for disease diagnosis is definitely not a new concept among the scientific and medical communities. Since Aristotle, doctors have been trained to use their sense of smell to detect odours from pathological origins, such as the faecal breath characteristic of liver disease, or the stale beer smell exhaled by patients with tuberculosis (Loudon, 1994). In the exhaled breath, the main olfactible molecular substrates are VOCs, which have been recurrently associated as biomarkers of several lung diseases, including asthma (van der Schee *et al*., 2015b).

2.2.1 The pathophysiological origin of exhaled volatile organic compounds

To comprehend the potential of exhaled VOCs as biomarkers of lung diseases, it is important to understand their origin and how they relate to the pathology. The human volatilome (the VOCs released from normal metabolic processes) is utterly complex, with a compendium of 872 compounds identified in the exhaled breath alone, in the healthy human (de Lacy Costello *et al*., 2014). These VOCs usually originate from normal microbiome metabolism or during the physiological processes of the liver, kidneys, and pancreas. After formation, the VOCs are either further oxidized into smaller components due to enhanced activity of enzymes, or directly conducted through the bloodstream, being subsequently excreted in the exhaled breath (Miekisch *et al*., 2004, Buszewski *et al*., 2007, van de Kant *et al*., 2012).

However, when a pathological condition is present, different patterns of VOCs may be released during the biochemical pathways of the inflammatory process, oxidative stress or changes in the human microbiome. In asthma pathophysiology, the chronic airway inflammation process and oxidative stress are responsible for the volatilome changes detected in the exhaled breath (Jiang *et al*., 2014). In general, oxidative stress is caused by reactive oxygen species (ROS) produced during chemotaxis of inflammatory cells and apoptosis resultant from the inflammation process. These ROS may leak from the mitochondria into the cytoplasm, subsequently promoting the degradation of

polyunsaturated fatty acids constituting lepidic structures, such as the bronchial epithelium cells membrane, resulting in the formation of volatile hydrocarbons (Mlakar and Spiteller, 1994, Rufo *et al*., 2016). These metabolites may either directly enter the blood stream as simple hydrocarbon molecules, such as alkanes, or be further oxidised by cytochrome P450 oxidase enzymes, leading to the production of alcohols, ketones or aldehydes, that are then released through the circulatory system (Filipiak *et al*., 2016). Finally, these pathologically originated compounds are excreted among the normal volatilome in the exhaled breath, which will generate different breathomic profiles when compared to a "healthy breath" (van de Kant *et al*., 2012). Figure 1 illustrates the pathological origin of exhaled VOCs in individuals with asthma.

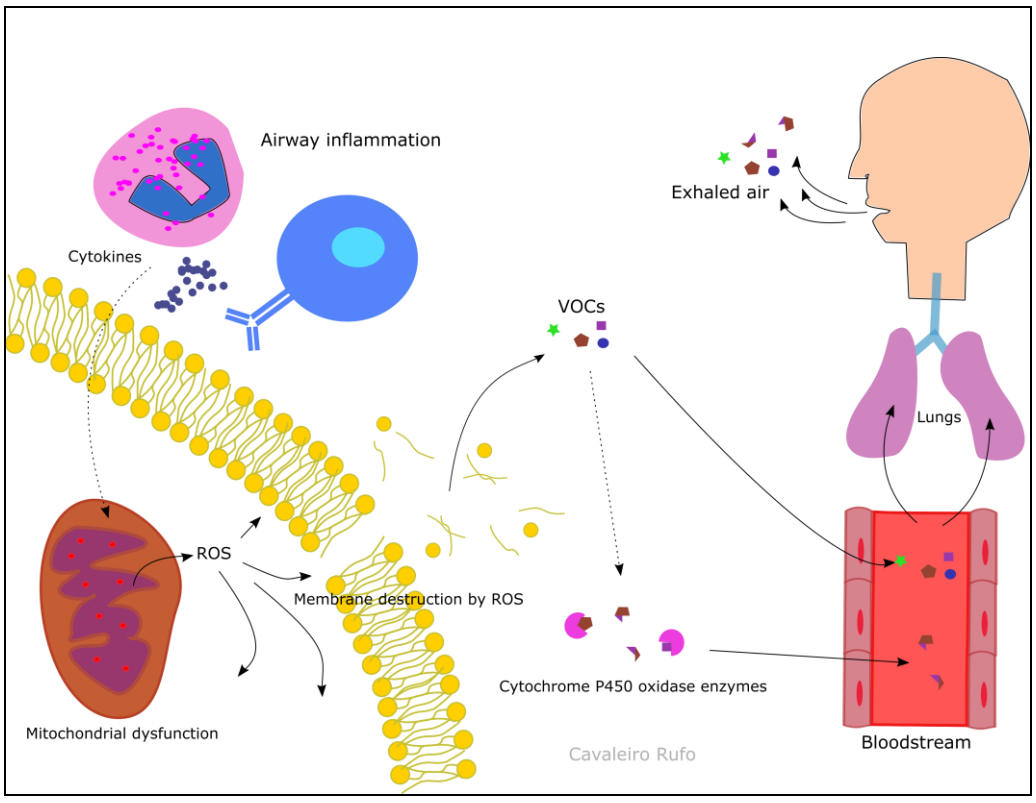

Figure 1 – Schematic representation of the production pathway for VOCs associated with asthma pathophysiology. Cytokines produced during airway inflammation in asthma may cause mitochondrial dysfunction in airway epithelial cells, promoting the release of reactive oxygen species (ROS). This ROS may cause depolarization of the lepidic structures in the cellular membrane, releasing volatile hydrocarbons that may directly enter the bloodstream, or be further oxidised cytochrome P450 oxidase enzymes, before being transported to the lungs and released in the exhaled breath.

Several studies have shown that individuals with asthma may be accurately distinguished from those without asthma by analysing their *breathprints* (exhaled VOC

profiles) (Dallinga et al., 2010, Montuschi et al., 2010, Ibrahim et al., 2011, Smolinska et al., 2014).

Since collecting breath is a simple, inexpensive and non-invasive process, breathomics appears to be a particularly promising approach for clinical diagnosis, phenotyping and monitoring of asthma. However, similarly to what occurs with other omics technologies, those associated with breathomics have their relevant limitations.

2.2.2 Methodological benefits and barriers of exhaled breath testing

There are currently several available technologies to analyse human exhaled breath. The most commonly used and recognized technique to identify and, to a certain extent, quantify exhaled VOCs, is the mammalian nose. As previously described, humans may use their sense of smell to detect odours characteristic of certain pathologies. However, the applications of the human nose are limited since most diseases do not produce olfactible amounts of compounds to the human, or are only olfactible when the disease is already at a significantly severe state. Other mammalians are known to have improved olfaction capacities, allowing them to detect abnormal breathprints associated with pathological states. A well-known example is Oscar the cat, who was able to smell the imminent death of the patients attending a nursing home in Rhode Island (Dosa, 2007). Nevertheless, the mammalian nose is currently a considerably limited method for breath analysis and as since the 1970s been replaced in breathomics by more precise and effective technologies, such as gas-chromatography-based techniques or the electronic nose (Das *et al*., 2016). Most of these technologies have been tested for exhaled air analysis of asthma diagnosis and the respective studies have been systematically reviewed by our team (Rufo *et al*., 2016).

First, however, it is important to discuss sample collection methodologies for breathomics in asthma. There are two reported matrices for exhaled VOCs assessment: exhaled air in its gaseous form, and EBC. Exhaled air is the most frequently used matrix for asthma breathomics since it is rapidly and effortlessly collectible, and appears to provide reproducible results (Dallinga *et al*., 2010). Typically, exhaled breath is collected using a sample bag, which usually has a septum where a needle may be inserted during analysis. In order to avoid contamination from exogenous VOCs, samples are normally collected through Tedlar bags (the capacity varies from 1 to 10 L in published studies). The

bags themselves are relatively expensive, but may be reused after cleansing with at least 3 nitrogen flushes (Caldeira *et al*., 2011). Although collecting gaseous exhaled breath appears to be the simplest method for asthma breathomics, the samples occupy large storage volumes and need to be analysed in a maximum of 6 hours after collection (Mochalski *et al*., 2009), which is a considerable barrier in real clinical applications. Contrasting with gaseous exhaled breath, EBC collection takes more time to collect since it requires subjects to breath normally through a gas condensing device during 10 to 15 minutes and involves sampling processing and heating before analysis, possibly resulting in the loss of some VOCs (Hüttmann *et al*., 2011). However, samples can be stored and frozen in glass tubes until analysis, allowing shipping and examination of multiple samples at once, which is an important advantage in the clinical diagnosis of highly prevalent diseases, such as paediatric asthma. Moreover, analysing the condensate sample itself does not appear to produce as good results as gaseous exhaled breath (Izquierdo-García *et al*., 2011, Carraro *et al*., 2013), although Hüttmann *et al*. (2011) showed that it is still possible to heat the condensate samples in order to release and measure VOCs in gaseous form.

Concerning assessment technology, gas-chromatography coupled to mass-spectrometry (GC-MS) has been consistently described as a good method for breathomics, and has been the most commonly used methodology for distinguishing breath samples of individuals with asthma in studies (Dallinga et al., 2010, Ibrahim et al., 2011, Caldeira et al., 2012). This technology not only allows the identification of compounds in a gaseous mixture, but also their relative quantification. Several studies showed improved diagnostic performances with enhanced versions of the GC-MS system, such as using two sequential chromatographers coupled to a time-of-flight mass-spectrometer (GCxGC-ToF-MS), as shown by Caldeira *et al.* (2011), allowing the distinction of children with allergic asthma from healthy controls and being able to identify the most discriminative compounds. A different approach was presented by Schivo and co-workers (2013), who used GC coupled to differential mobility and mass spectrometry (GC-DMS-MS) to successfully distinguish patients with asthma from controls. A summary of studies using GC-based technologies for asthma diagnosis is presented in table 3.

Table 3 – Summary of studies using GC-based techniques for exhaled breath analysis in asthma diagnosis. Adapted from Rufo *et al.* (2016b) with authorization from the copyright holder.

Reference	Objective	Participants	Breath collection	Technique
(Olopade *et al*., 1997)	Asthma diagnosis; Asthma severity monitoring	12 subjects with acute asthma, 11 subjects with stable asthma and 17 healthy controls	Tedlar bag (50 mL sampled)	GC-FID
(Paredi *et al*., 2000)	Asthma diagnosis; Asthma severity monitoring	26 subjects with asthma and 14 healthy controls	Tedlar bag (2 mL sampled)	GC-FID
(Delfino *et al*., 2003)	Asthma severity monitoring	21 subjects with asthma	Evacuated stainless steel canister	GC-MS
(Lärstad *et al*., 2007)	Asthma diagnosis	13 subjects with asthma and 14 healthy controls	Tedlar bag (3 L sampled)	GC-FID
(Dragonieri *et al*., 2007)	Asthma diagnosis; Asthma severity monitoring	10 subjects with severe asthma, 10 subjects with mild asthma and 20 controls	Tedlar bag (1 L sampled)	GC-MS
(Dallinga et al., 2010)	Asthma diagnosis	63 children with asthma and 57 healthy controls	Tedlar bag (5 L sampled)	GC-MS
(Montuschi *et al*., 2010)	Asthma diagnosis	27 subjects with asthma and 24 controls	Tedlar bag (2 L sampled)	GC-MS
(Caldeira *et al*., 2011)	Asthma diagnosis	35 children with asthma and 15 healthy controls	Tedlar bag (1 L sampled)	GC-MS
(Ibrahim *et al*., 2011)	Asthma diagnosis	35 subjects with asthma and 23 healthy controls	3 L collected directly in adsorbent pipes (Tenax)	GC-MS
(Gahleitner *et al*., 2013)	Asthma diagnosis	11 subjects with asthma and 12 healthy controls	2.5 L collected directly in adsorbent pipes (Tenax)	GC-MS
(Robroeks *et al*., 2013)	Asthma severity monitoring (prospective study)	40 children with asthma	Tedlar bag (5 L sampled)	GC-MS
(Schivo *et al*., 2013)	Asthma diagnosis; Differential diagnosis (Asthma vs. COPD)	13 subjects with asthma, 5 subjects with COPD and 13 healthy controls	Exhaled breath condensate collector (10 to 15 minutes)	GC-DMS-MS
(Smolinska *et al*., 2014)	Asthma diagnosis (Randomized trial)	76 children with asthma, 121 children with transient wheezing and 50 controls	Tedlar bag (1 L sampled)	GC-MS

GORD – Gastro-oesophageal reflux disease; GC-MS – Gas chromatography coupled with mass spectrometry; GC-FID – Gas chromatography coupled with flame ionization detector; GC-DMS-MS – Gas chromatography coupled to a differential mobility spectrometer and a mass spectrometer.

Although fairly sensible and able to identify a large part of the relevant compounds in a breathprint, GC-based techniques usually take a considerable amount of time per sample analysis (from 30 minutes to 2 hours per sample), require multiple resources, highly trained personnel, generally occupy large spaces and are completely dependent of transporter gases, meaning that gas tanks are needed in the proximity of the chromatographer (Krilaviciute *et al*., 2015, Neerincx *et al*., 2017). These limitations, in combination with the fact that no real-time findings can be obtained, impede point of care measurements to be conducted, or even the implementation of a properly equipped GC laboratory in clinical environments. Additionally, the technology is expensive and therefore difficult to execute in real clinical applications.

Ion mobility spectrometry (IMS) is another reported method for breath analysis. Shortly, exhaled VOCs are ionized and transported through drift tubes using a gradient created by electromagnetic fields. The ions then reach a detector in different drift times, in accordance with their characteristic mobility values (Buryakov *et al*., 1993). Although the rapid time-varying electromagnetic fields do allow identification of compounds in real-time, IMS is still short on accuracy, reproducibility and accurate cross-validation results for asthma diagnosis (Zrodnikov and Davis, 2012).

Another possible method for breath analysis is nuclear magnetic resonance (NMR). Unlike GC-MS and IMS, which may be used to analyse VOCs released from any exhaled breath matrix, NMR has only been performed in EBC matrices so far (de Laurentiis *et al*., 2008). This analytical technique creates a spectrum through the characterization of the most discriminant proton-containing low-molecular-mass compounds, providing a metabolic fingerprint of the analysed sample. Carraro and co-workers (2007), in a study comprising 25 children with asthma and 11 healthy children, were able to correctly identify 86% of the asthma cases using NMR. However, Izquierdo-García *et al.* (2011) contradicted these results, showing that NMR techniques do not have the sensitivity required to observe the endogenous metabolites presented in an EBC matrix. In addition, the complex analysis of the spectrograms, the immense dimensions of the spectrometer, prolonged acquisition times and overall cost, renders NMR as the least attractive method to implement in clinical breathomics.

Contrasting with GC and NMR techniques, the electronic nose (eNose) technology does not identify specific VOCs. Instead, the integrated cross-reactive nonspecific sensor arrays are exposed to VOC mixtures and generate a breathprint through pattern recognition

algorithms (Wilson and Baietto, 2011). The system can therefore compare and distinguish different smells without identifying the exact compounds in each breathprint (Wilson, 2015, Neerincx *et al*., 2017). Although GC-MS continues to be the preferred method for breathomics in experimental research due to its VOC identification capacity, the promptness, portability, relatively low cost and overall convenience, presents the eNose as the most appealing technology for point of care applications. Therefore, and since it appears to be the suitable method to rapidly analyse the exhaled breath of a large population of individuals with paediatric asthma attending clinical settings, the eNose technology will be further discussed in the next section.

2.2.3 Exhaled breath analysis by electronic nose

The eNose technology offers modern, non-invasive and cost-effective approaches to improve clinical diagnosis through exhaled breath analysis. Nevertheless, eNose in breathomics is a recent reality and still needs to overcome some challenges to finally being introduced in a real clinical scenario. Some of these challenges include the incapacity to identify individual compounds in complex VOC mixtures, relatively short sensor lifetime, slightly lower sensitivity than GC-MS and occasional problems of sensor translation (Wilson and Baietto, 2009). On the other hand, eNose instruments have many advantages over traditional analytical tools for breathomics, some already mentioned in the previous section, including being less expensive, relatively easy to use, rapid sampling and sensor recovery times, good precision, low operating costs and higher portability (Wilson, 2015).

There are many types of eNose instruments with different inherent sensing methods, ranging from surface acoustic wave sensors (Gan *et al*., 2005), quartz crystal microbalance arrays (Ali *et al*., 2003), metal oxide polymers (Green *et al*., 2011), to nanocomposite sensor arrays (Lorwongtragool *et al*., 2012). One of the most used instruments in biomedical applications is the commercially available Cyranose® 320 (Sensigent, California, USA) which relies on a nanocomposite sensor array comprising 32 vapour-sensitive sensors for creating breathprints from the measured VOC mixtures. The method of operation consists in setting baseline resistance values for the sensors by sampling ambient air, and then drawing the selected sample which will create different resistance values according to the VOCs in the mixture. Sensor deflection value in Cyranose® 320 is calculated according to the formula presented in equation 1.

Equation 1 – Calculation of sensor deflection value according to Cyranose® 320 method of operation.

$$R_{T0} = Sensor\ resistance\ at\ baseline$$

$$R_S = Sensor\ resistance\ during\ sampling$$

$$\Delta R = R_{T0} + R_S$$

$$Sensor\ deflection\ value = \frac{\Delta R}{R_{T0}}$$

There are already some published studies showing the potential of Cyranose® 320 in asthma diagnosis (Table 4). Dragonieri *et al.* (2007) showed that, in a clinical study comprising 20 individuals with asthma and 20 healthy controls, the breathprints of patients with asthma were fully separable from healthy controls, suggesting an important eNose diagnosis potential. This has been corroborated in a study conducted in the Netherlands, by Fens and co-workers (2009), where the authors aimed to correctly distinguish breathprints in a population comprised by 30 subjects with chronic obstructive pulmonary disease (COPD), 20 subjects with asthma and 40 healthy controls, by analysing their exhaled breath through Cyranose® 320. The results showed that breathprints of individuals with asthma were significantly different from those with COPD or with no respiratory diseases (controls) with an accuracy of respectively 96 and 95%. The external validation of the mentioned results was demonstrated in a later study by Fens *et al.* (2011), where they showed that the accuracy for discriminating asthma from COPD was not confounded by "current smoking" status.

Another interesting aspect of the eNose technology is its capacity to differentially diagnose individuals with added conditions suffering from the same disease (Rufo *et al.*, 2016). For instance, Timms and co-workers (2012) performed a study aiming to distinguish patients suffering from asthma with and without concomitant gastro-oesophageal reflux disease, using the Cyranose® 320 system. The population included 20 individuals with asthma and the results showed that breathprints from individuals with gastro-oesophageal reflux disease were highly distinguishable from those without reflux, in the asthma population. Moreover, eNose may prove to be an important tool to assist in asthma treatment administration, since van der Schee *et al.* (2013) found that exhaled VOC profiles were able to predict steroid responsiveness in patients with asthma with greater accuracy than exhaled NO and sputum eosinophils count, in a cross-sectional study comprising 25 subjects with asthma and 20 controls.

Table 4 - Summary of studies using eNose-based techniques for exhaled breath analysis in asthma diagnosis. Adapted from Rufo *et al.* (2016b) with authorization from the copyright holder.

Reference	Objective	Participants	Breath collection	Instrument
(Dragonieri *et al*., 2007)	Asthma diagnosis; Asthma severity monitoring	10 subjects with severe asthma, 10 subjects with mild asthma and 20 controls	Tedlar bag (1 L sampled)	Cyranose® 320
(Fens *et al*., 2009)	Differential diagnosis (Asthma vs. COPD)	30 subjects with COPD, 20 subjects with asthma and 40 controls	Tedlar bag (10 L sampled)	Cyranose® 320
(Fens *et al*., 2011)	Differential diagnosis (Asthma vs. COPD)	40 subjects with COPD and 60 subjects with asthma	Tedlar bag (10 L sampled)	Cyranose® 320
(Timms *et al*., 2012)	Differential diagnosis (Asthma with GORD vs. Asthma without GORD)	17 subjects with COPD, 20 subjects with asthma and 7 healthy controls	Tedlar bag (no volume specified)	Cyranose® 320
(van der Schee *et al*., 2013)	Asthma diagnosis; Steroid responsiveness prediction	25 subjects with asthma and 20 controls	Inert bag (not specified)	Cyranose® 320

Unfortunately, there are still no published studies showing the potential of eNose technology in the ever so challenging paediatric asthma diagnosis. The closest research on this matter has been performed by van der Schee *et al.* (2015), where the exhaled breath profile of 97 asymptomatic children was successfully discriminated from those of 81 wheezing children with an AUC of 0.77. Moreover, although presenting high sensitivity values for asthma diagnosis in adults, most of these studies lack stability measurements and external validation methods, fail to blind the diagnostic standard before performing breath analysis, and almost all of the studies use gaseous exhaled breath as the VOC collection matrix. As previously mentioned, exhaled air samples occupy large volumes (at least 1L per sample has been reported) and must be analysed in 6 hours after collection to avoid contamination of the sample bags (Mochalski *et al*., 2009). In addition, sample bags are expensive and, although reusable, they must be cleansed with multiple nitrogen flushes before each collection, thus being dependent of unpractical gas tanks (Caldeira *et al*., 2011). All these limitations would hamper the successful implementation of the eNose technology in real clinical diagnostics. Therefore, there is an emphasised need to investigate the accuracy of eNose in paediatric asthma diagnosis and, at the same time, develop a more practical method to allow large scale exhaled breath analysis in real clinical settings.

3 Aims of the thesis

The overall objective of the present thesis was to provide further insights on the development and diagnosis of paediatric asthma.

More specifically, the thesis programme aimed to:

1. Assess determinants of paediatric asthma in children-occupied environments, such as schools (study **I**) and indoor swimming pools (study **II**).

2. Evaluate the accuracy of exhaled VOC analysis in asthma diagnosis (study **III**).

3. Develop, validate and evaluate a breathomics model based on eNose exhaled breath condensate analysis for paediatric asthma diagnosis (study **IV**).

4 Materials and methods

This thesis is based on three different study designs:

1. A cross-sectional survey to observe how the prevalence of allergic sensitization and asthma in schoolchildren is influenced by the exposure to indoor air bacteria and fungi in classrooms, and by parasympathetic dysautonomia resultant from swimming pool exposure (studies I and II).
2. A systematic review and meta-analysis of published methodologies regarding exhaled VOC analysis for diagnosis and phenotyping of asthma (study III).
3. A cross-sectional study to evaluate the accuracy of a practical non-invasive eNose-based VOC analysis methodology for paediatric asthma diagnosis, using EBC matrices (study IV)

4.1 Study design and participants

4.1.1 Determinants of respiratory and allergic diseases in children-occupied environments (studies I and II)

A total of 20 public primary schools located in the city of Porto, Portugal, were invited to participate in this cross-sectional survey, corresponding to a total of 71 classrooms. The study was authorized by the Ethics Committee for Health of S. João Hospital Centre and by the schools' Directive Councils. Parents and legal guardians of 1602 children attending the participating schools were contacted and sent written information concerning the project. A written consent was retrieved for 916 children (57.2% participation rate), but only 858 completed the clinical assessment as the remaining 58 children refused to perform the clinical tests despite the legal guardians' consent (Figure 2). Therefore, and considering a confidence level of 95%, the estimated confidence interval for the sample size was 2.42. For convenience, the aforementioned sample of individuals shall be referred as "school population" throughout the thesis.

Indoor air sampling and health assessment campaigns occurred during the heating season in two different periods: from January to April 2014, and from October 2014 to March 2015 (10 schools per period). The heating season was chosen since the air exchange rates in schools are usually lower during this period. Throughout the sampling, indoor air bacteria, fungi and lipopolysaccharides (LPS) were collected.

Concurrently with the indoor sampling, the clinical assessment of the participating children was performed in the respective school. Height, weight, lung function (spirometry with bronchodilation) and exhaled NO levels were measured in participating children. Skin-prick-tests and pupillometry were also performed on the participants by a trained professional. A standardized ISAAC-based self-reported questionnaire focused on their child's respiratory and allergic symptoms, as well as on swimming practice, was filled by the parents.

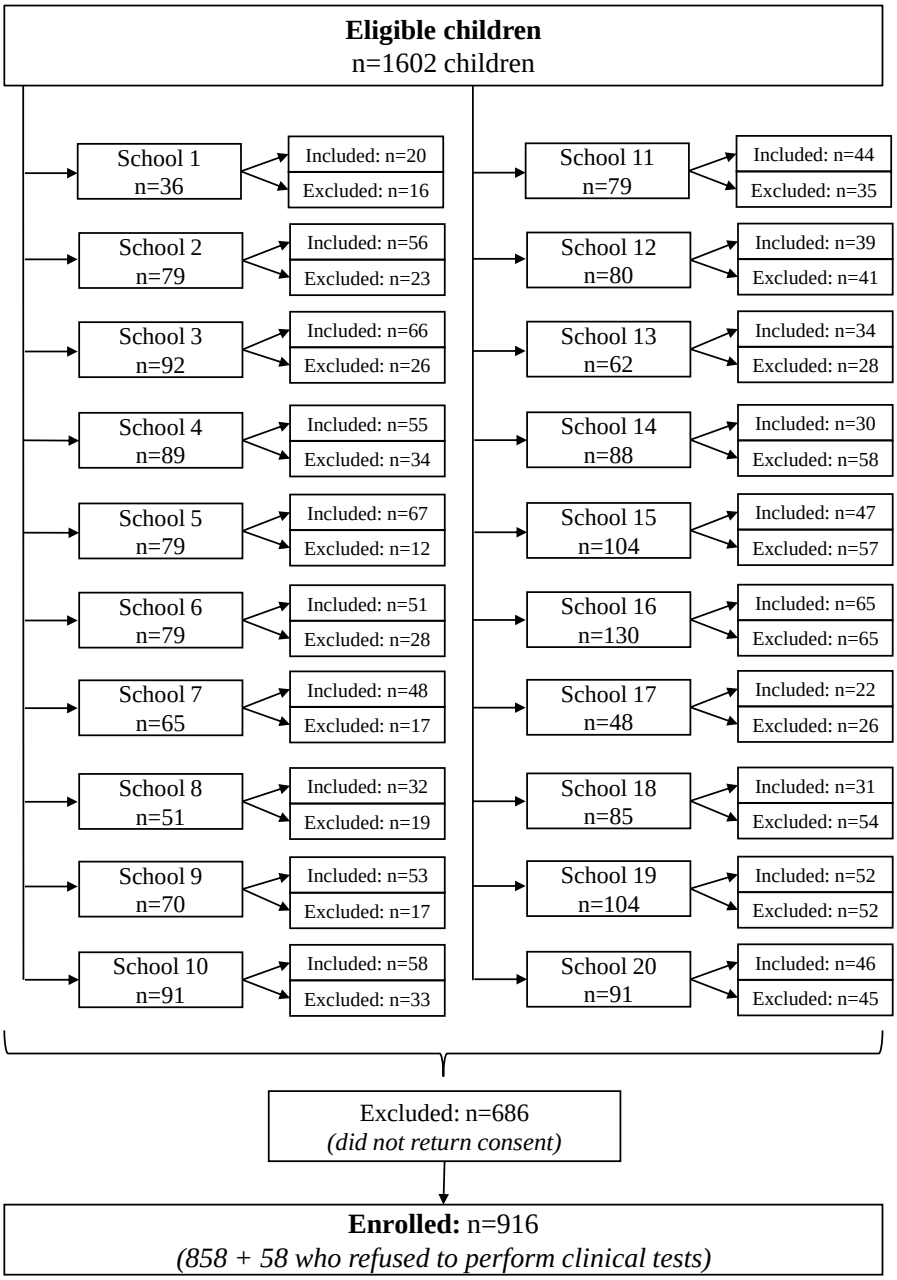

Figure 2 – Recruitment flow in the 20 participating schools (studies I and II). Reprinted from Cavaleiro Rufo *et al.* (2016) with authorization of the copyright holder.

4.1.2 Search strategy, inclusion criteria and data extraction (study III)

A PRISMA oriented systematic search (Moher *et al*., 2009) was performed until 31 October 2014 in PubMed, Scopus, Compendex, Inspec, ScienceDirect, Academic Search Complete, Web of Science and the Cochrane library. The search was conducted through the combination of the keywords "asthma", "exhaled", and "VOCs" or "volatile organic compounds". Published peer-reviewed full-text articles in English concerning clinical studies of asthma diagnosis through exhaled VOCs analysis were assessed for eligibility. The inclusion criteria for qualitative synthesis were (a) asthma defined by a trained physician or according to official guidelines, such as those validated by the Global Initiative for Asthma or the American Thoracic Society/European Respiratory Society; (b)VOCs measured in exhaled breath; and (c) clinical studies.

The exclusion criteria consisted in (a) less than two defined groups for comparing VOC levels or profiles; (b) studies focused on diagnosing symptoms of asthma, rather than the disease itself. Moreover, presenting the sensitivity and specificity values for asthma diagnosis was an additional inclusion criterion for the meta-analysis. Two reviewers independently applied the inclusion criteria and any differences were resolved by consensus.

Information regarding study design, settings, population, methodologies (including sample collection, analysis techniques, specific compounds and environmental air VOCs assessment) and outcomes was gathered. The tests' sensitivity and specificity data were retrieved whenever possible for meta-analysis.

The quality assessment of the selected studies was conducted according to the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) tool (Whiting *et al*., 2003). To better represent the quality assessment, QUADAS quality scores were defined: items classified as "Yes" added 1 point to the score; items classified as "No" and as "Unclear" added 0 points to the score (Attachment 1).

4.1.3 Recruitment design for the breathomics survey (study IV)

The cross-sectional study for accuracy testing followed the updated STARD guidelines for reporting diagnostic accuracy in studies (Bossuyt *et al*., 2015). Sample size estimation was based on previously reported accuracy measurements obtained through meta-analysis of 6 studies focused on exhaled VOCs in asthma diagnosis (Rufo *et al*.,

2016), and calculations were performed according to the classic likelihood ratio sample size estimation method for diagnostic test studies (Simel *et al*., 1991). Therefore, estimating a sensitivity of 87% and a specificity of 86% for asthma diagnosis for exhaled VOC analysis, and considering a sample comparison ratio of 1.0 for balanced judgment (disease vs non-disease), power calculations showed that 42 individuals with asthma and 42 individuals without asthma would be needed to show that the index test at least surpasses the 2.90 positive likelihood ratio achieved by spirometry with bronchodilation in asthma diagnosis, according to published data (Schneider *et al*., 2009), with a 95% confidence interval.

Participants, aged 6 to 18 years, were recruited from two distinct settings: during regular appointments to a tertiary care outpatient allergy clinic in S. João Hospital Centre, Porto, Portugal, from May to September 2016; and during a regular training session of a local juvenile football team in Porto, Portugal, between 5 and 13 September 2017. Eligible participants were recruited on a random basis, independently of the week day, time period of the visit, and appointed physician. After obtaining the legal guardians' informed consent and participant's agreement, airway reversibility measurements and SPT were performed, followed by EBC collection. Medical diagnosis of asthma and associated severity level (based on the administered treatment), as well as medical diagnosis of allergic rhinitis and atopic dermatitis, was established by an allergy specialist, randomly selected for each participant.

A total of 57 eligible individuals were invited to participate during the outpatient clinic visits, but only 51 were included in the study since 4 refused to partake, while the remaining 2 failed to produce valid EBC samples. During the football practice recruitments, all 13 elements of the football team that were eligible to participate accepted the invitation. Therefore, a total of 64 participants aged 6 to 18 years were included in the study. For convenience, this sample of individuals shall be referred as "breathomics population" throughout the thesis. Figure 3 summarizes the recruitment flow of participants.

The study was approved by the Ethics Committee for Health of S. João Hospital Centre (authorized at 14 April 2016) and by the National Data Protection Agency (nº 5057/2016).

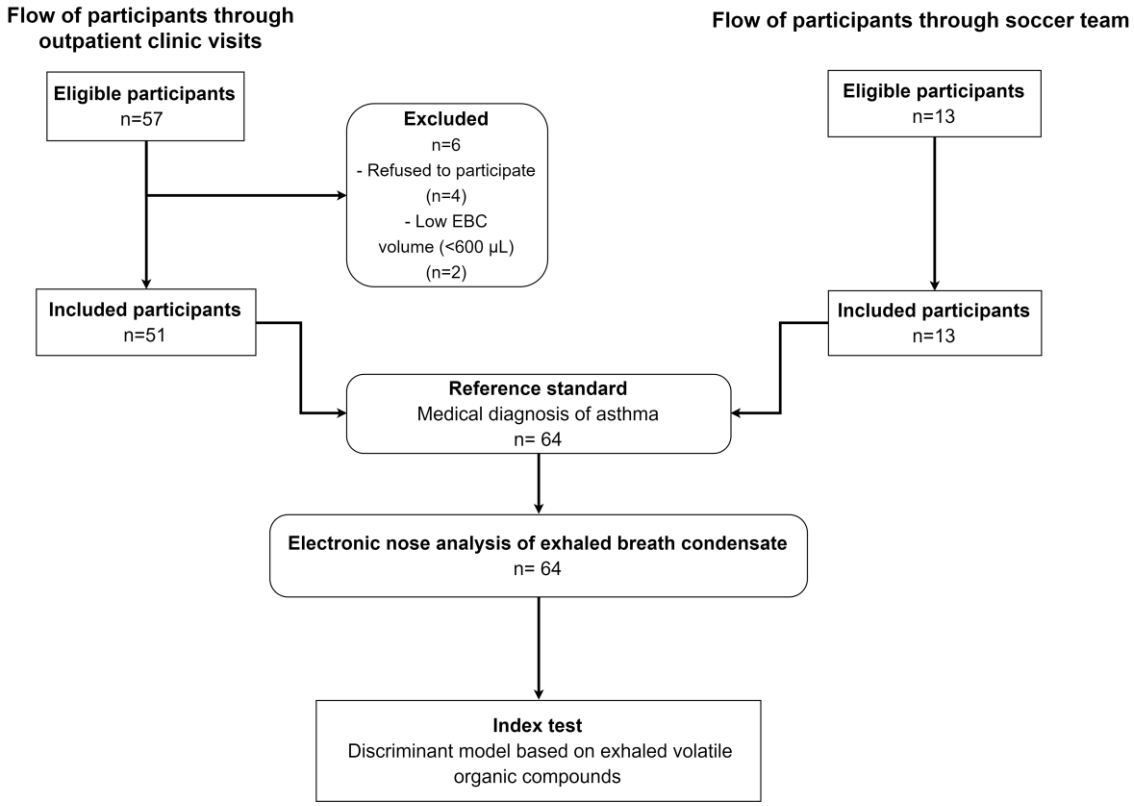

Figure 3 - Flow of participants through the study according to STARD (Bossuyt *et al.*, 2015).

4.2 Clinical and physiological assessments

A summary of the study outcomes and instruments is shown in Table 5.

Table 5 - Summary of study outcomes and biomarkers (studies I, II and IV). Medical diagnosis by an allergologist has also been performed in study IV.

Outcome	Biomarkers and instruments	Study	Reference
Lung function	Spirometry, Spirobank (MIR, Italy)	I, II, IV	(Miller *et al*., 2005)
Airway inflammation	Exhaled NO, NObreath (Bedfont Scientific Ltd., UK)	I, II	(Dweik *et al*., 2011)
Allergic sensitization	SPT, QuickTest™ applicator with allergen batch (Hall Allergy, Netherlands)	I, II, IV	(Global Initiative for Asthma, 2017)
Autonomic nervous function	Pupillometry, PLR-200™ Pupillometer (NeurOptics Inc, CA, USA)	II	(Muppidi *et al*., 2013, Stang *et al*., 2016)
History of symptoms	Standardized questionnaire based on ISAAC's	I, II	(Asher *et al*., 1995)
Exhaled VOCs	EBC, Turbo14 DECCS condenser system (Medivac, Parma, Italy)	IV	(Montuschi, 2007)

4.2.1 Physiological evaluation in schools (studies I and II)

Children's lung function and airway reversibility were assessed according to the ATS/ERS guidelines (Miller *et al*., 2005). Lung function data was retrieved before and 15 minutes after administering 400 μg of inhaled salbutamol.

Eosinophilic airway inflammation was assessed by measuring exhaled NO levels using the NObreath (Bedfont Scientific Ltd. UK). The results were expressed as parts per billion (ppb) and stratified according to the official ATS guidelines for children (Dweik *et al*., 2011).

Allergic sensitization was evaluated by SPT on their forearm using a QuickTest™ applicator and extracts of *Dermatophagoides pteronyssinus*, weed pollen mix (*Urtica dioica*, *Plantago lanceolate* and *Artemisia vulgaris*), grass pollen mix (*Agrostis stolonifera*, *Anthoxathum odoratum*, *Dactylis glomerata*, *Lolium perenne*, *Arrhenatherium elatius*, *Festuca rubra*, *Poa pratensis*, *Holcus lanatus*, *Phleum pratense*, *Secale cereal*), cat dander, dog dander and *Alternaria alternata*, negative control (extracts dilutant), and a positive control (histamine at 10mg/mL), all belonging to the same batches (Hall Allergy, Netherlands). Results were read 20 minutes afterwards. If children were on antihistamines or topical corticosteroids on the skin within the previous 7 days, SPTs were postponed.

Regarding pupillometry, the participants spent 15 minutes in a semi-dark and quiet room to allow their eyes to adjust to the low lighting levels before measurement. Pupillary measurements were taken with the portable infrared PLR-200™ Pupillometer (NeurOptics Inc, CA, USA). The complete pupillometry methodology has been thoroughly described in previous publications (Couto *et al*., 2015, Stang *et al*., 2016). Shortly, the pupil constriction response to a light stimulus represents parasympathetic activity, and the dilatation represents sympathetic activity. The following parameters were recorded: percentage of pupil constriction (CON); average and maximum constriction velocities (ACV and MCV, respectively); minimum and maximum pupil diameter; average dilation velocity (ADV); and the time in seconds at 75% recovery of pupil size (T75). Since there was no side-to-side difference in pupil responses, all pupillary data reported in the results was obtained from the right eye, in a similar approach to Muppidi *et al*. (2013). If a valid measurement was not obtainable, measurements from the left eye were used instead.

4.2.2 Clinical evaluation of the breathomics population (study IV)

For the paediatric population recruited during study IV, bronchodilation was stimulated by administering 400 μg of inhaled salbutamol, and post-bronchodilator spirometry was performed 20 minutes afterwards. Allergic sensitization was evaluated by SPT on the participant's forearm with extracts of *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, weed pollen mix, grass pollen mix, dog dander and *Alternaria alternata*, negative control (extracts diluent), and a positive control (histamine at 10mg/mL), all belonging to the same batches (CBF-LETI S.A., Madrid, Spain). Results were read 15 minutes afterwards. Information on medical diagnosis of asthma, rhinitis and atopic dermatitis was retrieved for each participant.

The Turbo 14 DECCS condenser system (Medivac, Parma, Italy) was used to collect EBC samples from included participants (Montuschi, 2007). The device was cooled to 0 ºC prior to collection, in accordance with manufacturer's instructions. EBC samples were obtained by at least 15 minutes of normal breathing while wearing a nose clip. Generally, 800 to 1500 μL of EBC was collected from each subject being a volume of at least 600μL stipulated as the minimal requirement for a valid sample. The different sample volumes are associated with the children's tidal and minute volumes of the lungs (Liu and Thomas, 2007).

After collection, samples were transferred to capped glass tubes and stored at -80 ºC until analysis. This procedure was performed in controlled environment through a laminar flow cabinet to decrease possible sample contamination by environmental air.

4.3 Definition of clinical and exposure outcomes

4.3.1 Clinical definitions for the school survey (studies I and II)

For the children recruited and evaluated at the 20 participating schools, the following operational asthma definitions were adopted: *i) Clinical criteria* – at least a 12% and over 200mL increase in FEV_1 after bronchodilation and/or self-report of asthma diagnosed by a physician with reported symptoms (wheezing, dyspnoea or dry cough) occurring in the past 12 months; *ii) Functional criteria* – at least a 12% and over 200mL increase in FEV_1 after bronchodilation; *iii) Treated asthma* – self-report of asthma diagnosed by a physician

and currently under inhaled corticosteroid treatment; and *iv) Ever asthma* – self-report of asthma diagnosed by a physician.

Allergic sensitization was defined by a positive SPT to at least one of the tested allergens (wheal > 3mm) coupled to a positive histamine response (wheal > 3mm) and no positivity in the negative control (wheal < 3mm) (Global Initiative for Asthma, 2017).

Atopic eczema was defined as a positive answer to the question "Did your child ever had itchy skin alterations that appeared and disappeared for at least 6 months, during the past 12 months?" followed by a positive answer to "Did these skin alterations ever affected elbow and knee joints, ankles, between thighs, or around the neck, ears or eyes?", based on the UK Working Party diagnostic criteria for the definition on atopic eczema (Williams *et al*., 1994). Otitis definition was based on a positive answer to the question "Did your child had ear pain or otitis in the last 12 months not associated with a cold or a flu?", while allergic rhinitis was defined as a positive answer to the question "Did your child suffer from recurrent sneezing, runny nose or nasal congestion, in the past 12 months, not associated with a cold or a flue?".

4.3.2 Reference standard for the breathomics survey (study IV)

Information on medical diagnosis of asthma, rhinitis and atopic dermatitis were retrieved for each participant of the breathomics population. The reference standard was the medical diagnosis of asthma, based on the spirometry with bronchodilation challenge results, as well as symptoms history and physical examination, according to the Global Initiative for Asthma guidelines (Global Initiative for Asthma, 2017).

Participants diagnosed with asthma and under maintenance inhaled steroid therapy were classified as having persistent asthma, while individuals with asthma simply under reliever inhaled therapy were regarded as having intermittent asthma. Medical diagnosis of allergic rhinitis and atopic dermatitis was based on the allergic sensitization results as well as symptoms history and physical examination.

4.3.3 Swimming pool attendance and extracurricular sports (study II)

According to the questionnaire answers, subjects were defined as current swimmers (CS) if parents answered "Yes" to the question: "Does your child swim frequently in an

indoor swimming pool (at least once per week)?"; if they answered "No, but he/she used to", participants were classified as past swimmers (PS); otherwise, if they answered "No", they were regarded as non-swimmers (NS). For the CS and PS groups, cumulative swimming pool exposure, expressed in years, was calculated. The question "Does your child partake in any sport activity outside of normal school-period, at least once per week?" was used to scrutinize if non or past swimmers practiced any other type of extracurricular sport, in order to exclude any conceivable bias associated with sedentarism or training volume. Figure 4 illustrates the recruitment flow and group selection criteria.

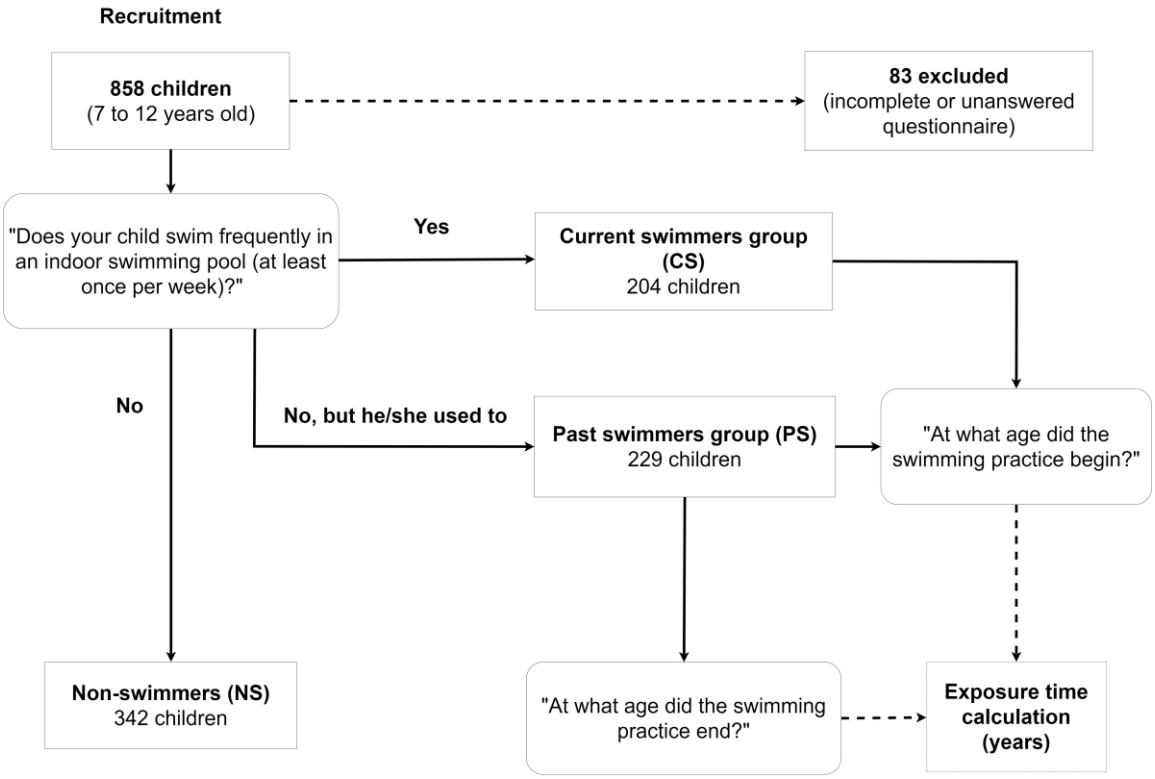

Figure 4 - Flow chart of the recruited participants (study II). Round-edged boxes represent the questions that allowed classification of participants into three different groups according to the respective answers (Cavaleiro Rufo et al., 2018).

4.4 Microorganism sampling (study I)

Bacterial and fungal air samples were collected in the 71 participating classrooms using a single-stage microbiological air impactor (Merck Air Sampler MAS-100), according to NIOSH method 0800 (National Institute for Occupational Safety and Health, 1998) and the European Standard 13098 (European Standards, 2000). Tryptic Soy Agar (supplemented with 0.25% cycloheximide) and Malt Extract Agar (supplemented with 1%

of chloramphenicol) were used as culture media for bacteria and fungi, respectively. Air was drawn through the sampler at 100 L/min and sequential duplicate air samples of 250 L were collected. A total of 2 Tryptic Soy Agar and 2 Malt Extract Agar samples were collected per classroom. The mean colony-forming unit (CFU) values of duplicate samples were used as the final result in accordance with laboratory criteria. The volume and, consequently, the duration of sequential air sampling was the same in all schools and classrooms. In each sampling day, four field blanks, two sterility blanks, one positive and one negative control per culture medium were used. This methodology has been validated in other publications (Madureira *et al*., 2014, Madureira *et al*., 2015).

Concurrently with bacteria and fungi assessment, indoor air LPS samples were collected during 4h with GilAir-5 flow control pumps (Sensidyne, USA) set to 2L/min and coupled to button aerosol stainless steel samplers (SKC, USA).

4.5 Laboratory procedures

4.5.1 Microbial analysis (study I)

The bacterial and fungal samples collected in classrooms were incubated at 37±1 ºC for 48±3 hours and at 25±3 ºC for 72±3 hours, respectively (Madureira *et al*., 2014, Madureira *et al*., 2015). Quantification of bacteria and fungi levels was performed by naked eye count following an internal procedure based on the methodologies expressed in European Standard 13098 (European Standards, 2000) and ISO 4833-1:2013 (International Organization for Standardization, 2013). The number of colonies recovered on the air-sample plates was adjusted using a positive-hole correction factor and the results were expressed as number of colony-forming units per cubic metre of air (CFU/m3). The correction factor was based on Fellers law. The quantification limit was established as 10 CFU per plate.

Specific fungal identification was performed 7 days after incubation, either on the original sampling medium plates or after subculturing procedures, whenever colony isolation and growth observation were needed. Identification of fungal colonies was based upon phenotypic characteristics and followed standard mycological procedures based on their micro and macro-morphological characteristics.

For endotoxin extraction, sampling filters were eluted in 5 ml extraction solution (Pyrogen Free Water plus 0.05% Tween 20) and rocked vigorously for 1 hour at room temperature on a horizontal shaker. After 10 minutes of centrifugation at 1000 g, total supernatant per sample was collected and analysed. Endotoxin quantification was performed using the limulus amebocyte lysate (LAL) Kinetic-QCLTM (Lonza®, Pontevedra, Spain) following the manufacturer's guidelines. Endotoxin concentrations were expressed as EU/m3. The limit of detection for the LAL Kinetic-QCLTM is 0.005 EU/mL, corresponding to 0.025 EU/m3 under the adopted procedure.

4.5.2 Exhaled VOC analysis by electronic nose (study IV)

To measure breathprints in EBC samples, the Cyranose® 320 (Sensigent, California, USA) eNose was used. This is a handheld device capable of detecting patterns of VOCs through 32 chemical sensors based on conducting chemoresistors made from carbon black nanocomposites. More information on Cyranose® 320 may be found in the *exhaled breath analysis by electronic nose* section of the literature review (section 2.2.3). The settings used for the current study are presented in Table 6.

Table 6 - Cyranose® 320 settings for EBC analysis.

Setting	Time	Speed
Baseline purge	10s	Medium
Sample draw 1	10s	Medium
Sample draw 2	0s	*n.a.*
Snout removal	10s	*n.a.*
1st sample gas purge	20s	High
1st air intake purge	30s	High
2nd sample gas purge	20s	High
2nd air intake purge	0s	*n.a.*

n.a.: not applicable

Samples were defrosted at ambient temperature prior to processing. The whole measurement procedure was conducted in a laminar flow cabinet to prevent ambient air

contamination and the cabinet's temperature and relative humidity were continually measured. For VOC analysis, 600 µl of EBC was transferred to 12x75mm glass assay tubes which were then covered with laboratory film. The tubes were subsequently heated in dry bath at 37 ºC for 2 minutes to increase the gas phase and an exhaust was created in the laboratory film, as previously described (Hüttmann *et al.*, 2011). Afterwards, a 20 g needle was attached to the Cyranose® 320 snout and used to pierce the laboratory film while holding the device above the surface. The laminar flow cabinet's air was used as reference air while baselining for 10 seconds and then a sample was drawn for another 10 seconds. This procedure was repeated for all samples.

4.6 Construction of a breathomics model (study IV)

The 32 sensor resistance values provided by the eNose were retrieved and imported as a dataframe object to the R software v3.4.2 (R Foundation, Vienna, Austria) through the RStudio interface v1.0.153 (RStudio INC., Massachusetts, USA). In a first step, principal component analysis (PCA) was applied for exploratory observation of the data dimension and distribution (Figure 5).

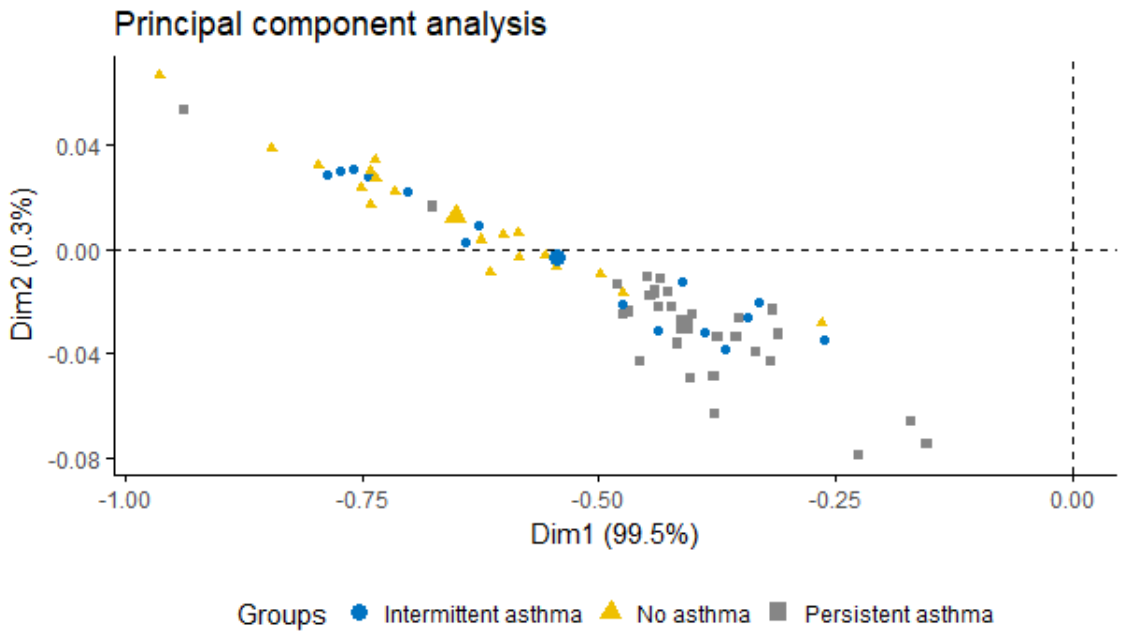

Figure 5 – Spatial distribution of the eNose sensor resistance values based on two components, after principal component analysis.

To assess clustering tendency, the Hopkin's index was calculated and a dissimilarity matrix was drawn (Figure 6). Since the Hopkin's statistic retrieved a value of 0.89, data was considered as highly clusterable (Banerjee and Dave, 2004).

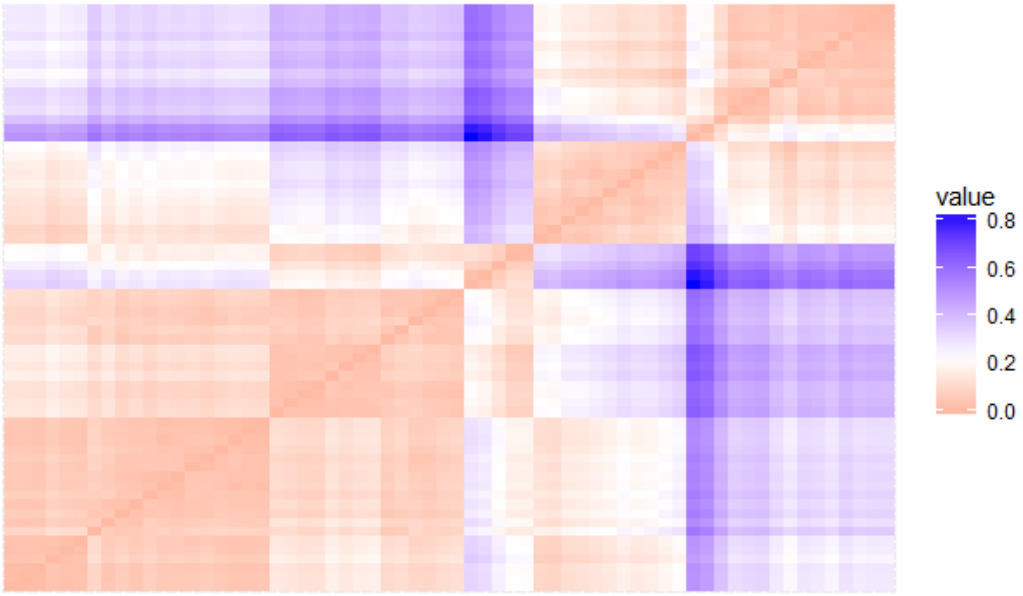

Figure 6 – Dissimilarity matrix of the eNose sensor data. The agglomerated colours suggest highly clusterable data.

Internal validation and cluster stability methods were performed and optimal scores where achieved in both cases through hierarchical clustering with k=2. To further confirm the optimal number of clusters, the total within sum of squares and the average silhouette width methods were applied. Both methods confirmed the optimal cluster score of k=2 (Figure 7 and 8). Therefore, a hierarchical model with two clusters was created using the 32 sensor resistance values, blinded to the reference standard results (Figure 9).

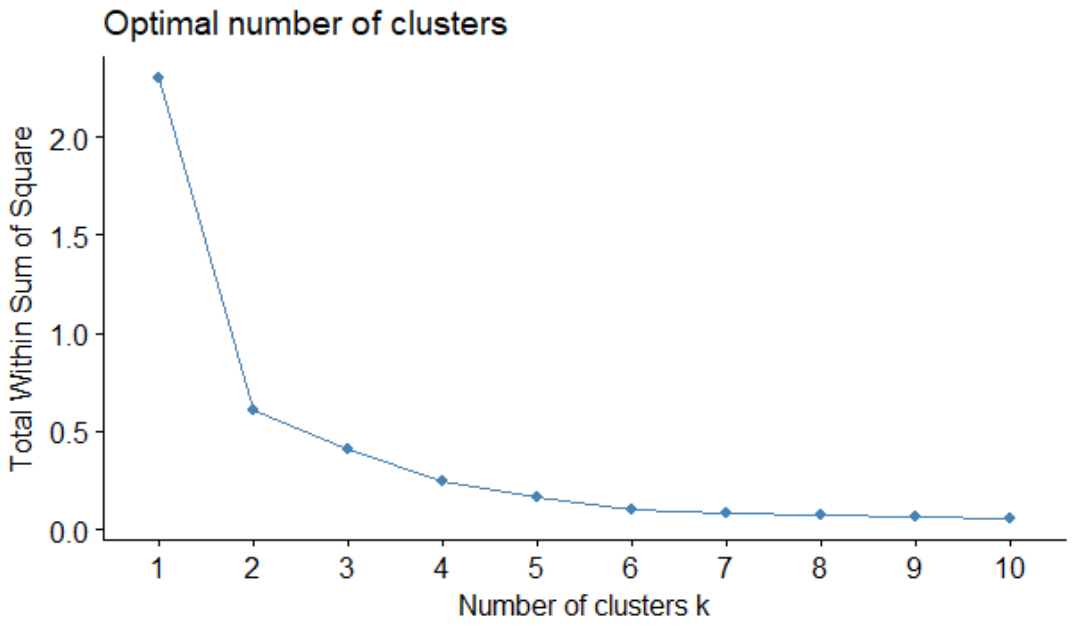

Figure 7 - Total within sum of squares (elbow) method for determining optimal number of clusters. The "elbow" of the curve indicates the suitable number of clusters for a given dataframe. In this case, the elbow bends at k=2, suggesting that data may be grouped in two different clusters.

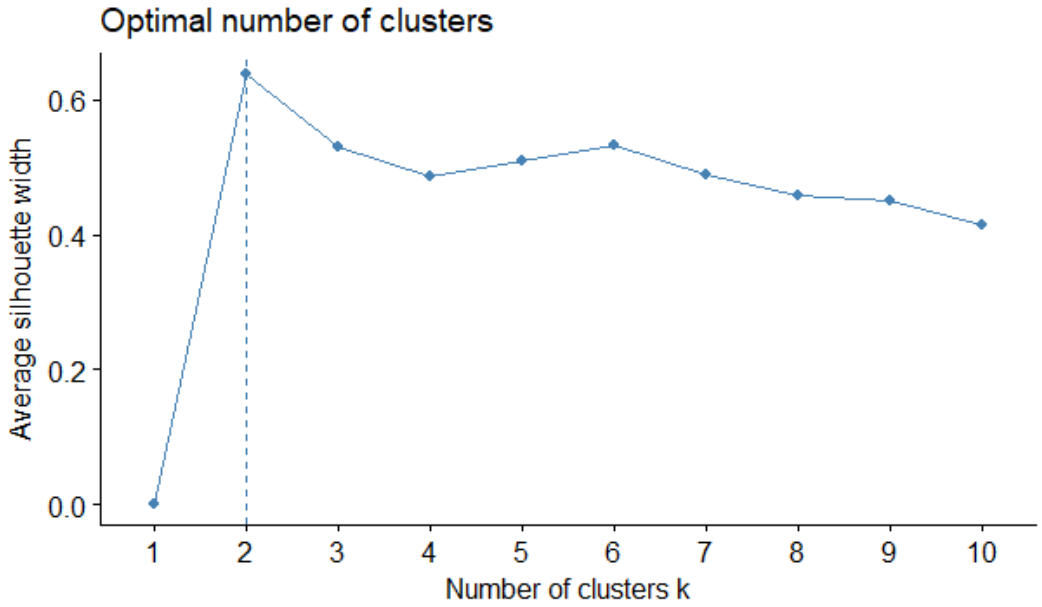

Figure 8 - Silhouette method for determining optimal number of clusters. The peak of the curve indicates the suitable number of clusters for a given dataframe. In this case, the curve peaks at k=2, suggesting that data may be grouped in two different clusters.

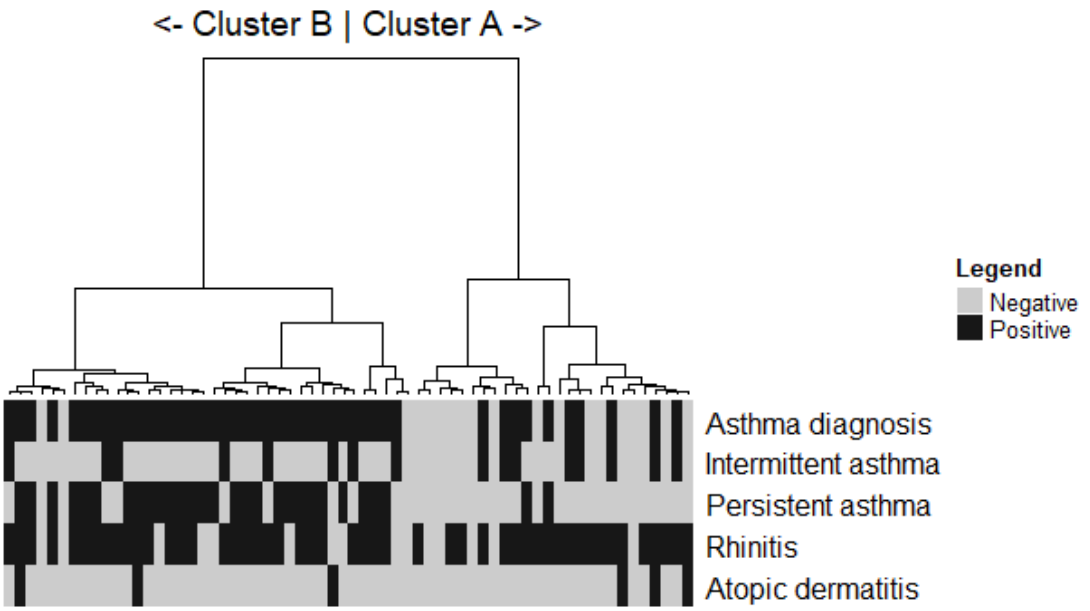

Figure 9 – Heatmap representing data divided in two clusters (A and B) based on hierarchical multivariate analysis.

4.7 Data processing and statistical analysis (studies I – IV)

The SPSS® statistical package software v20.0 (IBM, New York, USA) was used to statistically analyse the data in study I and II, as well as to perform t-student and chi-square tests between the two clusters of the hierarchical model, conducting discriminant analysis, and calculate the accuracy, sensitivity, specificity and areas under ROC curves in study IV. The meta-analysis in study III was performed using the Stata IC software v13.0 (StataCorp LP, Texas, USA) and the Meta-DiSc software v1.4 (Clinical BioStatistics Unit, Madrid, Spain) (Zamora *et al*., 2006).

The Kolmogorov-Smirnov test was used to check continuous variables for normality in studies I and II. In study IV, the Shapiro-Wilk test was used instead due to the smaller sample size. Whenever non-Gaussians distributions were observed, non-parametric tests were used for inferential analysis. Significant differences were reported with an α-value inferior to 5% ($p<0.05$), when applicable.

When analysing the specific fungi species in study I, each species' proportions were generally categorized into terciles. Since in some cases the detected levels were very low, such as the case of *Acremonium spp*, the variable was dichotomised with the lower category consisting in values under the limit of detection, while the higher category included those over the detection limit. A microbial diversity score was defined as the sum

of all detected fungi groups/species in a given classroom. The variable was categorized into quartiles, being the classrooms with lower diversity score in the 1st quartile, whereas the 4th quartile included those with higher diversity scores. Logistic regression adjusted for age and height was used to analyse the risk for allergic sensitization and asthma associated with the collected indoor air microbiology (total and specific values). Multinomial logistic regression was used for analysing the risk of inflammation reported by exhaled NO. The results were expressed as odds ratio (OR) and respective 95% confidence interval (95% CI).

To analyse the data concerning study II, the t-student test and one-way ANOVA (for normal distributions) or Mann-Whitney and Kruskal-Wallis tests (for non-parametric distributions) were used to compare continuous variables between two or more than two groups of individuals, respectively. The Spearman's correlation test was used to find correlations between the number of years in swimming practice and the measurable outcomes. Logistic regression analysis was then used to find associations between the number of years in swimming practice and the binary categorical variables, with results expressed in β [95% CI].

In the meta-analysis of the systematic review (study III), the sensitivity and specificity of the included studies were used to construct a contingency table. The bivariate meta-analysis model was employed to obtain the pooled value of sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (DOR) and generate the bivariate summary ROC curve. Heterogeneity between studies was evaluated using the chi-square and I^2 tests. As previously mentioned, a value of $p<0.05$ was considered as being statistically significant, while $I^2 \geq 50\%$ indicated the existence of significant heterogeneity (Higgins et al., 2003, Dinnes et al., 2005). For the purpose of threshold effect detection, and to evaluate the relationship between sensitivity and specificity, the Spearman's correlation test was used. Publication bias of the selected studies was assessed using Begg's funnel plot and Egger's test (Borenstein *et al*., 2009).

T-student test was used to find if there were differences in participants' characteristics between groups, for continuous variables, in study IV. Chi-square tests were used for inferential statistics between two categorical variables and risk estimation was performed to assess the tendency of asthma severity according to each cluster of the hierarchical model. Discriminant analysis was performed to identify the most relevant variables in the model. Receiver operating characteristic curves were built and accuracy, sensitivity,

specificity and AUC values were calculated according to standardized methods (Baratloo *et al*., 2015, Carter *et al*., 2016).

4.8 Ethics

The studies' protocols were authorized by the Ethics Committee for Health of S. João Hospital Centre (studies I, II and IV), the schools' Directive Councils (studies I and II), and by the National Data Protection Agency (study IV). The recruitment process of study IV was registered in the Clinical Trials platform under the registry number "NCT02802891". Parents or legal guardians of participating children (studies I, II and IV) provided their signed informed consent, while subjects that were aged 18 (study IV) signed their own.

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5 Results

5.1 Participants

5.1.1 Children assessed at school (studies I and II)

The characterization of the participants recruited for studies I and II is presented in Table 7. Prevalence of asthma diagnosed by clinical criteria, functional criteria, treated asthma and ever asthma was 9.3, 6.6, 5.4, and 6.3%, respectively. Prevalence of allergic sensitization was 34.1%. The prevalence of allergic rhinitis and atopic dermatitis was 10.6 and 9.3%, respectively.

Table 7 – Characteristics of participating children evaluated at school (studies I and II).

	Total (n= 858)	**Female (n= 427)**	**Male (n= 431)**
Age (years, mean ± sd)	9 ±1	9 ±1	9 ±1
Weight (kg)	31.2 (27.3 – 37.2)	31.8 (27.0 – 37.3)	30.8 (27.5 – 36.8)
Height (cm)	135 (130 – 140)	135 (130 – 139)	135 (130 – 140)
BMI (kg/m^2)	17.0 (15.5 – 19.5)	17.3 (15.6 – 19.8)	16.8 (15.5 – 19.2)
Clinical parameters			
Allergic sensitization (n)	293	139	154
Allergic rhinitis (n)	91	37	54
Atopic eczema (n)	80	37	43
Asthma prevalence[β]			
Clinical criteria (%)	9.3	11.5	7.2
Functional criteria (%)	6.6	8.2	5.1
Treated asthma (%)	5.4	6.1	4.6
Ever asthma (%)	6.3	6.8	6.2
Lung function			
FVC (L)	1.89 (1.69 – 2.14)	1.83 (1.66 – 2.08)	1.94 (1.72 – 2.18)
FEV_1 (L)	1.75 (1.58 – 1.95)	1.71 (1.55 – 1.92)	1.77 (1.59 – 1.99)
FEV1/FVC (%)	92.7 (88.9 – 96.4)	93.2 (89.8 – 96.6)	92.0 (88.0 – 96.1)
FEF_{25-75} (L/s)	2.28 (1.92 – 2.65)	2.28 (1.93 – 2.64)	2.29 (1.91 – 2.68)
FEV_1 reversibility (%)	3.5 (0.0 – 7.1)	3.7 (0.0 – 7.2)	3.5 (0.5 – 7.0)
FEV_1 reversibility (ml)	60 (0 – 120)	60 (0 – 120)	60 (10 – 130)
Exhaled NO (ppb)	11.0 (6.0 – 20.0)	9.5 (5.0 – 16.0)	12.0 (6.0 – 22.5)

Data reported as median (25-75%) unless otherwise stated. BMI: body mass index; FEV_1: forced expiratory volume in the first second of FVC; FVC: forced vital capacity; FEF_{25-75}: forced expiratory flow middle portion of FVC. [β]The following operational asthma definitions were adopted: i) Clinical criteria – at least a 12% increase in FEV_1 after bronchodilation and over 200mL and/or asthma diagnosed by a physician with reported symptoms (wheezing, dyspnoea or dry cough) occurring in the past 12 months; ii) Functional criteria – at least a 12% increase in FEV1 after bronchodilation and over 200mL; iii) Treated asthma criteria – asthma diagnosed by a physician and currently under inhaled corticosteroid treatment; and iv) Ever asthma – asthma diagnosed by a physician.

In the framework of study II, the school population characterized above was further separated into three groups according to swimming practice, as described in the methodology section (4.3.3). Due to incomplete or unanswered questionnaires, 83 participants were excluded from the study. Therefore, a total of 205, 228 and 342 children were classified as CS, PS and NS, respectively (Table 8 and 9).

Table 8 – Physiological parameters of participants according to swimming pool attendance (study II).

Parameters	Current swimmers	Past swimmers	Non-swimmers	*p*
N (males)	205 (99)	228 (115)	342 (175)	- -
Age (years, mean ± sd)	8.7 ±0.8	8.6 ±0.7	8.9 ±0.8	**0.011**[¥]
Weight (kg)	30.9 (26.6 to 36.9)	32.1 (28.2 to 37.8)	30.8 (26.9 to 37.3)	**0.048**
Height (cm)	135 (130 to 139)	136 (131 to 141)	136 (131 to 141)	0.196
Sport practisers (%)	100.0	98.3	98.2	0.965
Lung function				
FVC (L)	1.88 (1.71 to 2.15)	1.91 (1.71 to 2.18)	1.88 (1.66 to 2.10)	0.125
FEV_1 (L)	1.73 (1.58 to 1.95)	1.78 (1.60 to 1.99)	1.74 (1.55 to 1.92)	0.081
FEV_1/FVC (%)	92.8 (89.1 to 96.1)	92.5 (89.0 to 96.6)	92.7 (88.8 to 96.4)	0.998
FEF_{25-75} (L/s)	2.23 (1.97 to 2.71)	2.36 (1.93 to 2.71)	2.27 (1.91 to 2.59)	0.423
PEF (L/s)	3.77 (3.30 to 4.38)	3.77 (3.38 to 4.21)	3.69 (3.27 to 4.27)	0.641
FEV_1 reversibility (mL)	70 (20 to 130)	60 (10 to 120)	60 (-10 to 110)	**0.028**
FVC reversibility (mL)	40 (-30 to 100)	30 (-30 to 80)	20 (-40 to 90)	0.219
Exhaled NO (ppb)	12 (7 to 20)	11 (6 to 20)	10 (5 to 19)	0.086
Asthma[β]				
Clinical criteria (n, %)	11.7%	8.3%	9.4%	0.459*
Functional criteria (n, %)	6.3%	7.0%	6.4%	0.957*
Treated asthma (n, %)	6.3%	4.0%	6.1%	0.441*
Ever asthma (n, %)	7.3%	4.8%	7.3%	0.438*
Atopic eczema (n, %)	66.7%	62.9%	54.4%	0.465*
Allergic rhinitis (n, %)	33.3%	33.8%	31.1%	0.907*
Allergic sensitization (%)	32.8	39.5	34.2	0.302*
Otitis (n, %)	27.0%	21.0%	32.4%	**0.043***

Data reported as median (P25 to P75) unless otherwise stated. BMI: body mass index; FEV_1: forced expiratory volume in the first second of FVC; PEF: Peek expiratory flow; FVC: forced vital capacity; FEF_{25-75}: forced expiratory flow middle portion of FVC; EBC: exhaled breath condensate. The *p* values signalling differences between the three groups were calculated using the Kruskal-Wallis test for non-parametric variables, with the exception of cases marked with (*) which were calculated using qui-square tests, and (¥), which were calculated using one-way ANOVA (for normal distributions).

[β]The following operational asthma definitions were adopted: i) Clinical criteria – at least a 12% increase in FEV1 after bronchodilation and over 200mL and/or asthma diagnosed by a physician with reported symptoms (wheezing, dyspnoea or dry cough) occurring in the past 12 months; ii) Functional criteria – at least a 12% increase in FEV1 after bronchodilation and over 200mL; iii) Treated asthma criteria – asthma diagnosed by a physician and currently under inhaled corticosteroid treatment; and iv) Ever asthma – asthma diagnosed by a physician.

Table 9 – Pupillometry parameters of participants according to swimming pool attendance (study II).

Pupillometry parameters	Current swimmers	Past swimmers	Non-swimmers	*p*
Maximum (mm, mean ± sd)	5.2 ±0.9	5.3 ±1.0	5.3 ±0.8	0.278
Minimum (mm, mean ± sd)	3.4 ±0.6	3.4 ± 0.6	3.4 ±0.6	0.537
CON (%, mean ± sd)	35 ±5	36 ±5	36 ±5	0.203
ACV (mm/s, mean ± sd)	3.8 ±0.7	3.9 ±0.8	4.0 ±0.7	**0.030**
MCV (mm/s, mean ± sd)	5.1 ±1.0	5.3 ±1.0	5.4 ±0.9	**0.010**
ADV (mm/s, mean ± sd)	1.2 ±0.3	1.2 ±0.4	1.2 ±0.3	0.709
T75 (s, mean ± sd)	1.7 ±0.7	1.7 ±0.7	1.7 ±0.7	0.987

CON: percentage of pupil constriction; ACV: average constriction velocity; MCV: maximum conscription velocity; ADV: average dilation velocity; T75: time in seconds at 75% recovery of pupil size
The *p* values signalling differences between the three groups were calculated using one-way ANOVA. Significant differences in bold.

5.1.2 Breathomics population in two clusters (study IV)

The exhaled VOC-based hierarchical model was able to significantly discriminate individuals with asthma from those without the disease, in the two clusters ($p<0.001$). In addition, persistent asthma cases, which were individuals who had been administered inhaled corticosteroid medication, were also significantly distinguished in the model ($p<0.001$). Consequently, 100% of positive bronchodilation challenges were reported in the same cluster ($p<0.001$). However, exhaled VOC analysis was unable to discern patients with allergic rhinitis and atopic dermatitis from those without these conditions ($p=0.511$ and $p=0.623$, respectively). A summary of these results is presented in Table 10.

Discriminant analysis showed that the highest correlation between the hierarchical model and standardized canonical discriminant functions was achieved by persistent asthma variable (rho=0.602), followed by spirometry with bronchodilation (rho=0.544). These results show that the analysed exhaled VOC profiles were hierarchised mainly according to the asthma severity status of the patients, with those needing inhaled medication (persistent asthma) presenting significantly different VOC profiles from those with intermittent asthma or without asthma at all (Table 11).

Table 10 - Differences between the hierarchical model clusters, created using the 32 sensor resistance values measured by the eNose, according to participants' characteristics.

Characteristics	Cluster A	Cluster B	*p*
n (males)	26 (17)	38 (24)	0,855
Age (years, mean ± sd)	10,7 (±3,5)	12,1 (±3,1)	0,223*
Height (cm, mean ± sd)	148,2 (±19,3)	155,2 (±15,2)	0,050*
Weight (kg, mean ± sd)	43,3 (±15,4)	51,4 (±14,9)	0,920*
Positive BD (n)	0	20	**<0,001**
Positive SPT (n)	22	29	0,418
Medical diagnosis of asthma (n)	10	35	**<0,001**
Intermittent asthma (n)	8	8	0,378
Persistent asthma (n)	2	27	**<0,001**
Allergic Rhinitis (n)	21	28	0,511
Atopic dermatitis (n)	3	3	0,623

p values in bold correspond to significant differences between clusters. Differences calculated through qui-square tests, except for continuous variables (marked with *), which were calculated through t-student tests.

Table 11 - Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions. Variables are ordered by absolute size of correlation within function.

Discriminating variables	rho*
Persistent asthma	0.602
Bronchodilation	0.544
Weight	0.156
Age	0.146
Height	0.134
Intermittent asthma	-0.077
Skin prick tests	-0.052
Allergic rhinitis	-0.042
Sex	0.035
Atopic dermatitis	-0.028
Recruitment setting	-0.009

*Correlation coefficient for standardized canonical discriminant functions

5.2 Influence of classroom microbial diversity on asthma and allergic sensitization risk in children (study I)

Classrooms with 2nd tercile (OR = 1.26 [95% CI: 0.88 – 1.79]) and 3rd tercile (OR = 1.40 [95% CI: 0.99 – 1.99]) concentrations of LPS showed a tendency for a higher risk of allergic sensitization (Figure 10A). Moreover, classrooms with higher concentrations of LPS had a significantly higher prevalence of allergic children (2.20 [0.89 – 4.25] vs 2.60 [1.18 – 5.68], data presented as median [25% - 75%], respectively non-sensitized vs sensitized, p=0.02). On the other hand, no tendencies were observed for asthma.

Diversity scores ranged from 1 to 11. Logistic regression models showed a negative association between the diversity of fungal species in classrooms and the risk of allergic sensitization (Figure 10B). These results were significant for the 3rd (0.63 [95% CI: 0.40 – 0.98]) and 4th (0.60 [95% CI: 0.40 – 0.92]) quartile scores, which ranged from 7 to 8.

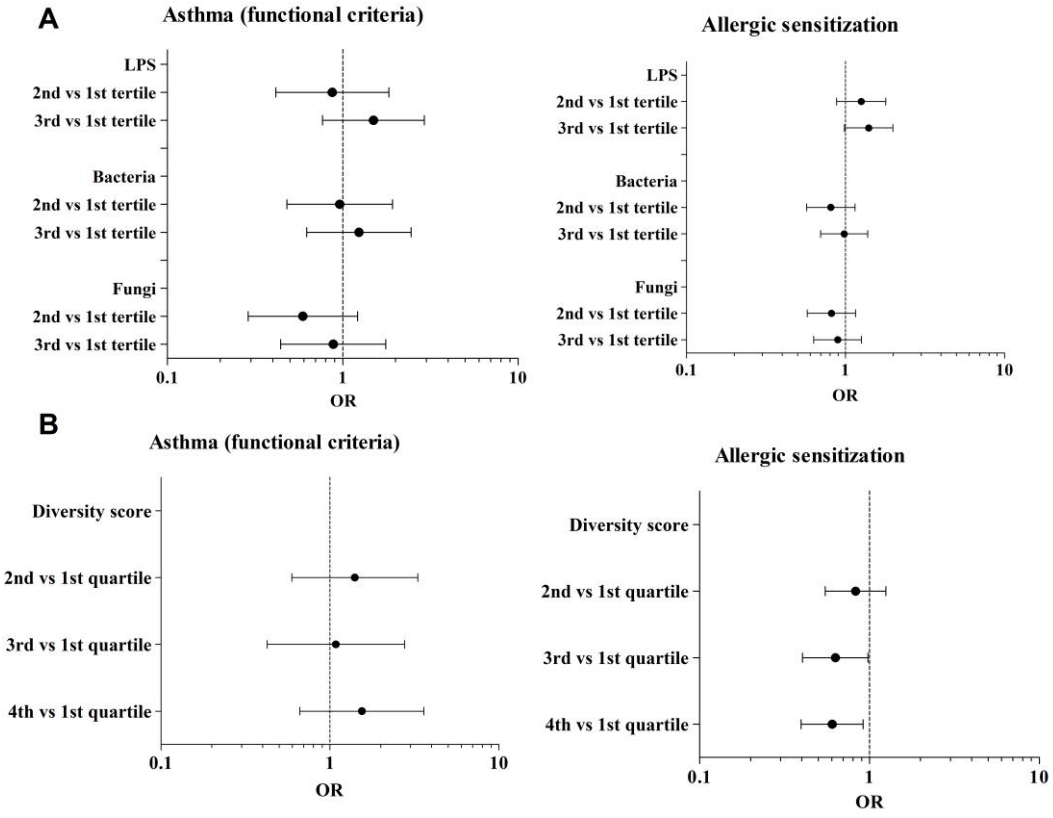

Figure 10 - A: Logistic regression between LPS, bacterial and fungal exposure in classrooms and the prevalence of asthma and allergic sensitization. B: Logistic regression between fungal diversity scores and the prevalence of asthma and allergic sensitization. The results are expressed as odds ratios with 95% CI in logarithmic scale.

Additional pro-sensitization evidence was observed with specific fungal species, as classrooms with 2nd tercile concentrations of *Penicillium spp* were associated with a higher risk of sensitization (OR = 1.46 [95% CI: 1.02 – 2.09]) and classrooms with 3rd tercile concentrations showed an even higher risk (OR = 1.68 [95% CI: 1.18 – 2.40]). This tendency for a higher sensitization risk associated with *Penicillium spp* can be observed in Figure 11. On the other hand, classrooms with higher concentrations of *Aspergillus fumigatus* (3rd vs 1st tercile, OR = 0.64 [95% CI: 0.47 – 0.87]), *Aspergillus niger* (detected vs not-detected, OR = 0.62 [95% CI: 0.45 – 0.87]), *Chaetomium spp* (detected vs not-detected, OR = 0.61 [95% CI: 0.39 – 0.96]) and *Rhizopus spp* (detected vs not-detected, OR = 0.62 [95% CI: 0.45 – 0.87]) were associated with a lower risk of allergic sensitization (Figure 11 and 12). Interestingly, the risk ratios were very similar between these species (children had 1.56 to 1.64 times less risk of being sensitized).

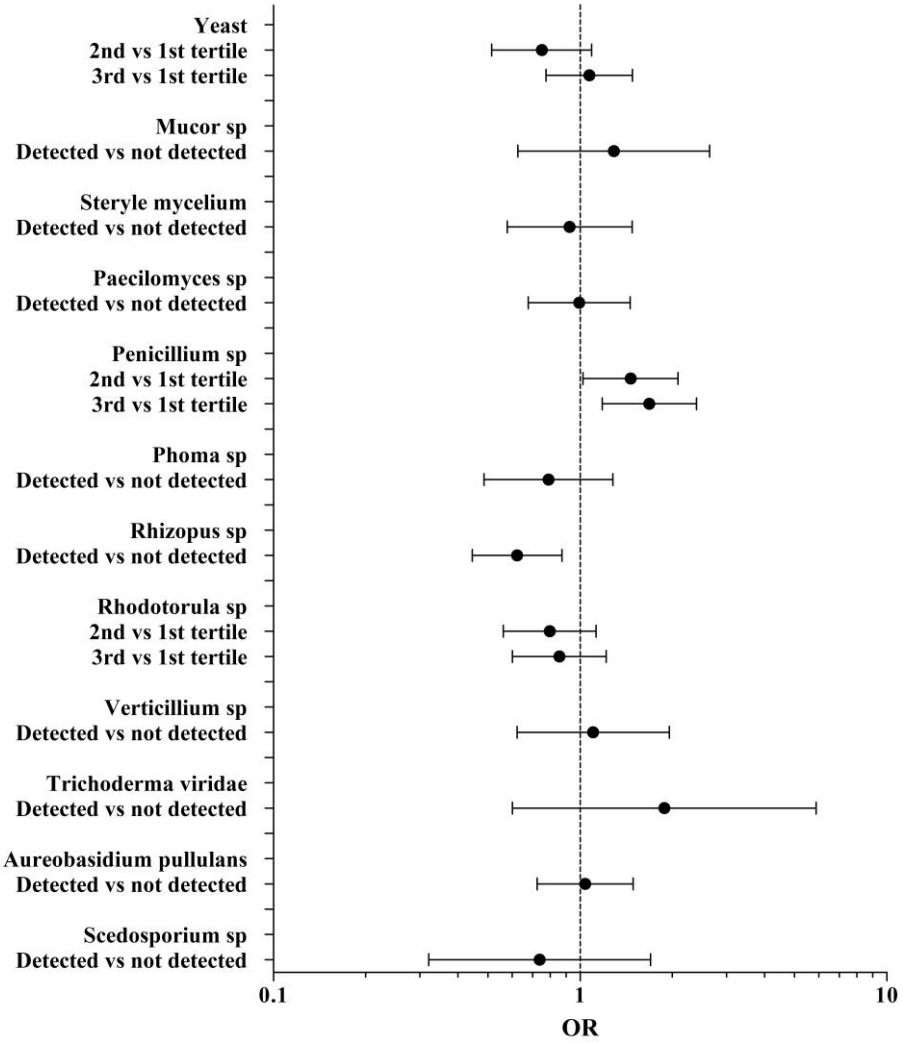

Figure 11 – How fungal species influence allergic sensitization (first set).

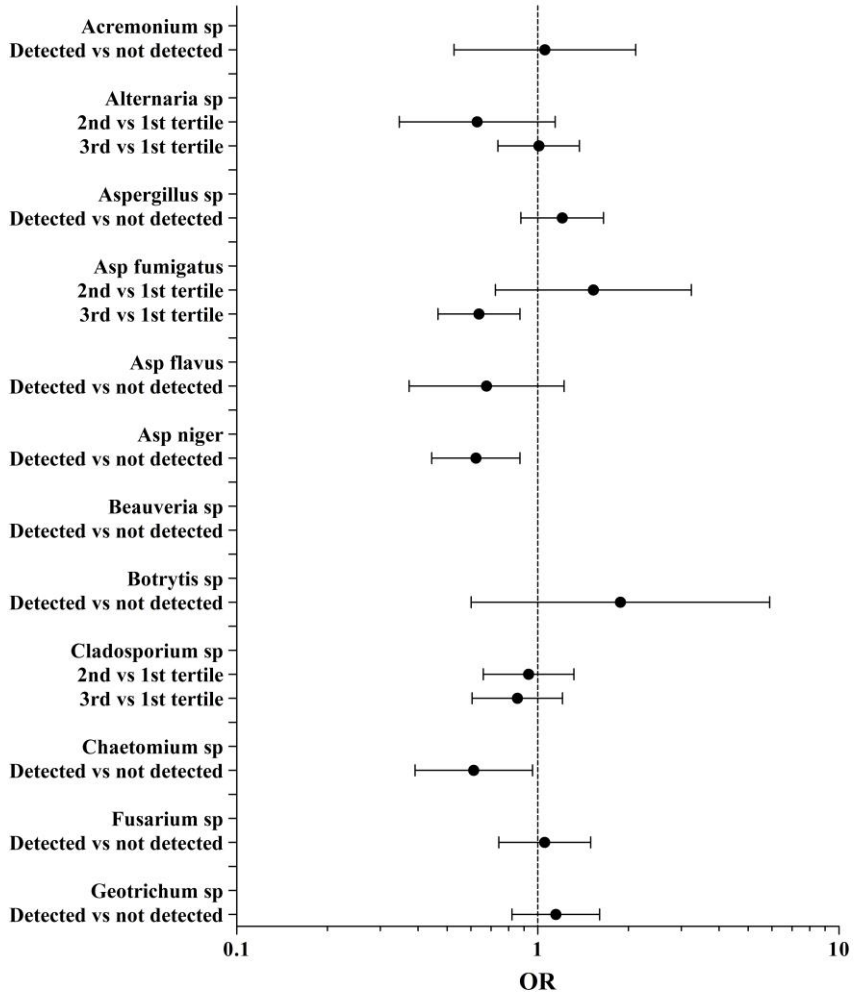

Figure 12 - How fungal species influence allergic sensitization (second set).

Unlike allergic sensitization, the classrooms' specific fungal species and flora diversity were not associated as a risk factor for any of the 4 different definitions of asthma (Figure 13). Moreover, the higher tendency observed for functional asthma was not replicated for the other definitions.

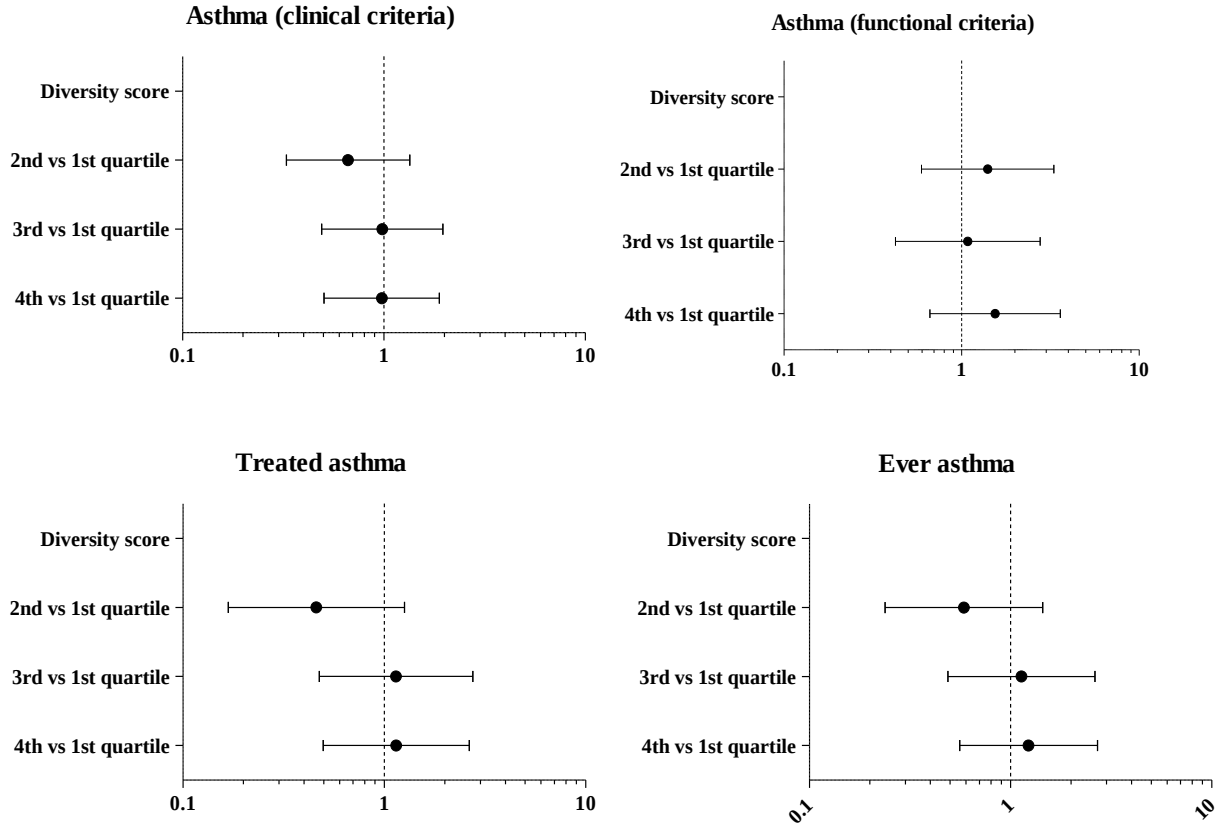

Figure 13 – The risk of asthma, using 4 definitions, associated with fungal diversity in classrooms. The following operational asthma definitions were adopted: i) Clinical criteria – at least a 12% increase in FEV1 after bronchodilation and over 200mL and/or asthma diagnosed by a physician with reported symptoms (wheezing, dyspnoea or dry cough) occurring in the past 12 months; ii) Functional criteria – at least a 12% increase in FEV1 after bronchodilation and over 200mL; iii) Treated asthma criteria – asthma diagnosed by a physician and currently under inhaled corticosteroid treatment; and iv) Ever asthma – asthma diagnosed by a physician.

5.3 Effects of swimming pool attendance in schoolchildren (study II)

Children in the CS group had significantly lower MCV and ACV when compared to both PS and NS groups, MCV (mm/s, mean±sd): 5.1 ±1.0 *vs* 5.3 ±1.0 *vs* 5.4 ±0.9, respectively (p=0.010); and ACV(mm/s, mean±sd): 3.8 ±0.7 *vs* 3.9 ±0.8 *vs* 4.0 ±0.7, respectively (p=0.030). Moreover, levels of exhaled NO and changes in airway reversibility volume after administration of a beta-2 agonist were significantly higher in the CS group when compared to NS (respectively, median [P25 to P75]: 12ppb [7 to 20] *vs* 10 ppb [5 to 19], p=0.030; and 70mL [20 to 130] vs 60mL [-10 to 110], p=0.007) (Table 8 and Figure 13). These results were not influenced by sedentarism and/or other sport practice

since no significant difference was found between percentage of weekly sport practisers between groups (p=0.965).

There were no differences between the three groups of participants for the prevalence of asthma (for any of the 4 definitions) or allergic sensitization, although a significantly higher occurrence of otitis was observed in NS when compared to PS (32.4 *vs* 21.0%, respectively; p=0.013), but not to CS (32.4 *vs* 27.0%, respectively; p=0.251).

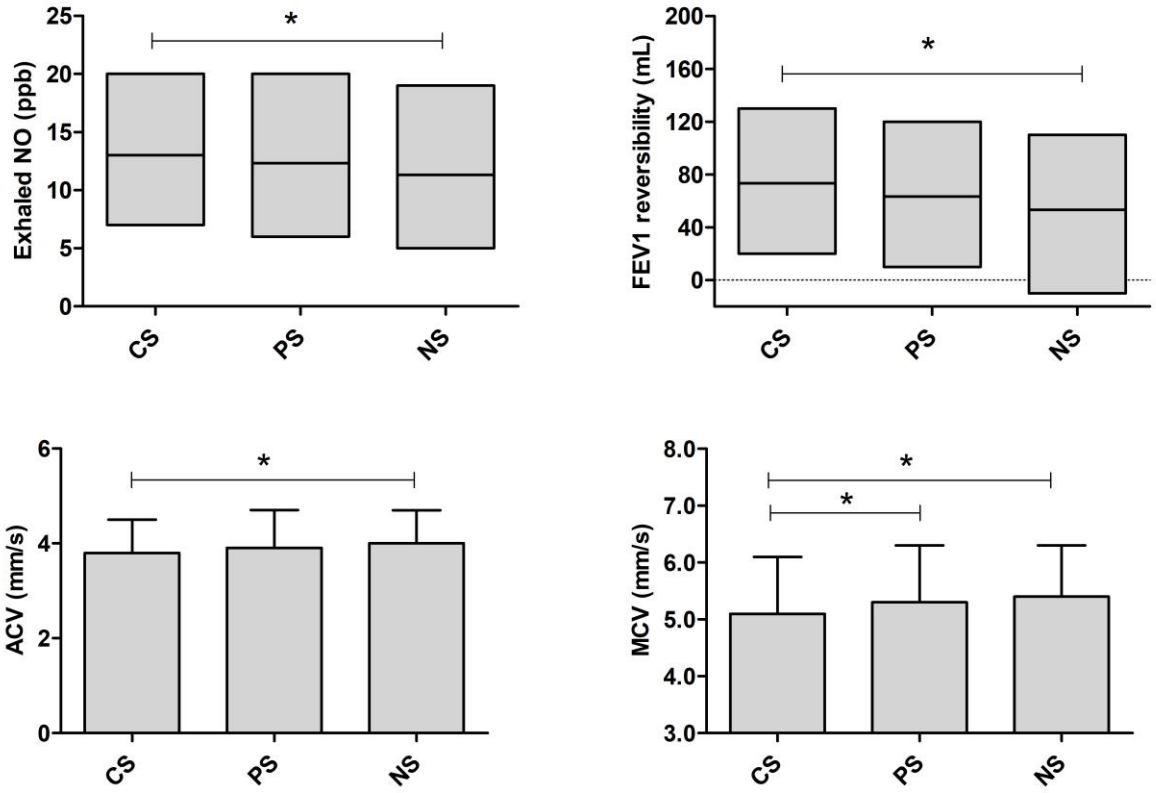

Figure 14 - Median (with 25 and 75 percentiles) levels of exhaled NO and FEV1 reversibility, and mean ±SD of measured ACV and MCV among the three groups. CS – current swimmers; PS – past swimmers; NS – non-swimmers. *Represents significant differences between two groups indicated by the extremities of the horizontal line (p<0.05) (Cavaleiro Rufo et al., 2018).

Differences found in the studied physiological outcomes were not influenced by the inclusion of individuals with asthma in the groups since, with the exception of atopic eczema, there were no significant changes when exclusively comparing individuals with clinical defined asthma (Table 12).

Table 12 - Clinical parameters of individuals with asthma, between the three groups. For this analysis, participants with asthma were selected according to the clinical criteria: at least a 12% increase in FEV_1 after bronchodilation and over 200mL and/or asthma diagnosed by a physician with reported symptoms (wheezing, dyspnoea or dry cough) occurring in the past 12 months.

	Current swimmers	Past swimmers	Non-swimmers	*p*
N (males)	24 (10)	19 (8)	23 (9)	- -
Age (years, mean ± sd)	8.9 ±1	8.7 ±0.7	8.8 ±0.8	0.640¥
Weight (kg)	33.1 (31.0 to 37.1)	32.3 (28.9 to 37.5)	30.7 (26.6 to 39.5)	0.514
Height (cm)	136 (133 to 140)	135 (132 to 141)	135 (129 to 138)	0.587
Sport practisers (%)	100.0	100.0	96.9	0.506
Allergic sensitization (%)	45.8	38.9	46.9	0.852*
Lung function				
FEV_1 (L)	1.69 (1.50 to 1.95)	1.72 (1.53 to 1.89)	1.64 (1.38 to 1.81)	0.608
PEF (L/s)	3.54 (3.27 to 4.38)	3.62 (3.17 to 3.77)	3.66 (2.86 to 3.93)	0.667
FVC (L)	1.87 (1.67 to 2.07)	1.95 (1.69 to 2.08)	1.86 (1.58 to 2.05)	0.473
FEF_{25-75} (L/s)	2.13 (1.67 to 2.79)	1.90 (1.62 to 2.62)	2.01 (1.63 to 2.59)	0.535
FEV_1/FVC (%)	92.8 (87.6 to 96.0)	87.1 (83.5 to 94.1)	92.2 (85.3 to 96.9)	0.283
FEV_1 reversibility (mL)	22 (5 to 27)	23 (21 to 33)	22 (11 to 30)	0.388
FVC reversibility (mL)	11 (3 to 23)	19 (3 to 28)	18 (2 to 24)	0.636
Exhaled NO (ppb)	20 (12 to 45)	15 (8 to 28)	20 (4 to 42)	0.572
Otitis (n, %)	35.3%	26.7%	30.8%	0.870*
Atopic eczema (n, %)	100%	83.3%	33.3%	**0.048***
Allergic rhinitis (n, %)	41.7%	50.0%	45.8%	0.916*
Pupillometry				
Maximum (mm, mean ± sd)	5.2 ±1.1	5.3 ±0.8	5.3 ±0.7	0.908¥
Minimum (mm, mean ± sd)	3.4 ±0.9	3.4 ± 0.5	3.4 ±0.5	0.932¥
CON (%, mean ± sd)	35 ±5	36 ±5	35 ±5	0.982¥
ACV (mm/s, mean ± sd)	3.6 ±0.8	3.9 ±0.7	4.0 ±0.7	0.166¥
MCV (mm/s, mean ± sd)	5.0 ±1.1	5.5 ±1.0	5.3 ±1.0	0.324¥
ADV (mm/s, mean ± sd)	1.2 ±0.3	1.0 ±0.2	1.1 ±0.3	0.146¥
T75 (s, mean ± sd)	2.0 ±0.7	1.7 ±0.9	1.7 ±0.6	0.444¥

Data reported as median (P25 to P75) unless otherwise stated. BMI: body mass index; FEV_1: forced expiratory volume in the first second of FVC; PEF: Peek expiratory flow; FVC: forced vital capacity; FEF_{25-75}: forced expiratory flow middle portion of FVC; EBC: exhaled breath condensate; CON: percentage of pupil constriction; ACV: average constriction velocity; MCV: maximum conscription velocity; ADV: average dilation velocity; T75: time in seconds at 75% recovery of pupil size.

The *p* values signalling differences between the three groups were calculated using the Kruskal-Wallis test for non-parametric variables, with the exception of cases marked with (*) which were calculated using qui-square tests, and (¥), which were calculated using one-way ANOVA (for normal distributions).

As expected, the number of years in swimming practice was significantly higher in CS when compared to PS (mean (±SD) = 3.9 (±2.2) vs 2.4 (±1.6), respectively; $p<0.010$). To investigate the effect of cumulative exposure resultant from swimming practice, correlations between the number of years in swimming practice and continuous variables of the measured health outcomes were calculated using the Spearman's correlation test (Table 13). Significant correlations were observed for baseline FEV_1 (rho=0.11), PEF (rho=0.18), and the CON (rho=0.12), ADV (rho= -0.13) and T75 (rho=0.19) parameters of pupillometry. Also, the logistic regression analysis showed a non-significant trend for a higher risk of asthma by lung function criteria (β [95% CI] = 1.13 [0.94 to 1.37]), atopic eczema (β [95% CI] = 1.15 [0.86 to1.53]) and allergic rhinitis (β [95% CI] = 1.10[0.89 to 1.36]) with more years of swimming practice (Figure 15).

Table 13 - Spearman's correlation test between continuous clinical parameters and the number of years in swimming practice. Values represent the Spearman's correlation coefficient. Significant correlations are expressed in bold.

	Number of years in swimming practice (rho)
Lung function parameters	
FVC	.098
FEV_1	**.111**
FEV1/FVC	-.001
FEF 25–75%	.054
PEF	**.184**
Forced expiratory flow	.008
FEV_1 reversibility	.064
Inflammatory parameters	
Exhaled NO	.016
Pupillometry parameters	
Maximum diameter	.014
Minimum diameter	-.030
CON	**.118**
ACV	.013
MCV	-.001
ADV	**-.126**
T75	**.186**

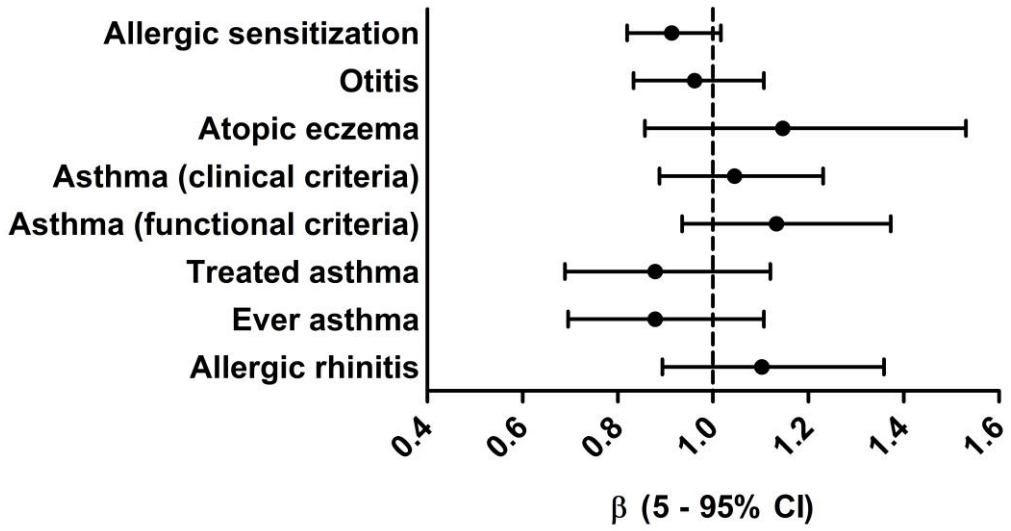

Figure 15 - Logistic regression between the number of years in swimming practice and the assessed clinical outcomes (adjusted for age). Results are represented as β (5-95%CI) (Cavaleiro Rufo et al., 2018).

5.4 Exhaled VOC analysis in asthma diagnosis (study III).

Six studies retrieved from the systematic review presented sufficient data to be included in the meta-analysis (Dallinga et al., 2010, Montuschi et al., 2010, Ibrahim et al., 2011, Caldeira et al., 2012, van der Schee et al., 2013, Smolinska et al., 2014). Sensitivity and specificity of exhaled VOC analysis in asthma diagnosis tests were retrieved from these studies and grouped according to the methodology used. For the purpose of the meta-analysis, the methodology of analysis used (eNose or GC-MS) was not discriminated since it only represents different means to produce similar outcomes for diagnosing asthma through exhaled VOCs. Data retrieved from the aforementioned studies is presented in Table 14.

The sensitivity and specificity values of the exhaled VOC analysis test for asthma diagnosis were analysed. Heterogeneity in specificity was observed among the 6 studies (I^2=65.1%; p=0.014), which did not occur with sensitivity (I^2=46.6%; p=0.095). Therefore, the random effects model was employed. The pooled results reported a mean (95% CI) sensitivity of 87% (82% to 91%) and specificity of 86% (80% to 90%). The mean (95% CI) pooled PLR was 5.86 (3.07 to 11.21), indicating that individuals with asthma had approximately 6 times higher chance of presenting asthma-associated exhaled VOC profiles when compared with individuals without asthma, and the mean (95% CI) pooled

NLR was 0.16 (0.10 to 0.26). The AUC was 0.94 and the mean (95% CI) pooled DOR was 49.3 (15.9 to 153.3) (Figure 16 and 17, respectively).

Table 14 - Studies included in the meta-analysis.

Reference	Technique	Sample size	Accuracy	Sensitivity	Specificity
(Montuschi *et al*., 2010)	Metalloporphyrins coated QMB sensor	12 subjects with asthma vs 12 controls (analysis in alveolar exhaled breath)	88%	100%	75%
(van der Schee *et al*., 2013)	Cyranose® 320	25 subjects with asthma and 20 controls (post-steroid comparison)	82%	84%	80%
(Dallinga et al., 2010)	GC-MS	63 children with asthma vs 57 healthy controls	92%	89%	95%
(Ibrahim *et al*., 2011)	GC-MS	35 subjects with asthma vs 23 healthy controls	86%	85%	89%
(Caldeira *et al*., 2012)	GC-MS	32 children with allergic asthma vs 27 healthy controls	98%	96%	95%
(Smolinska *et al*., 2014)	GC-MS	76 children with asthma vs 50 healthy controls	77%	82%	74%

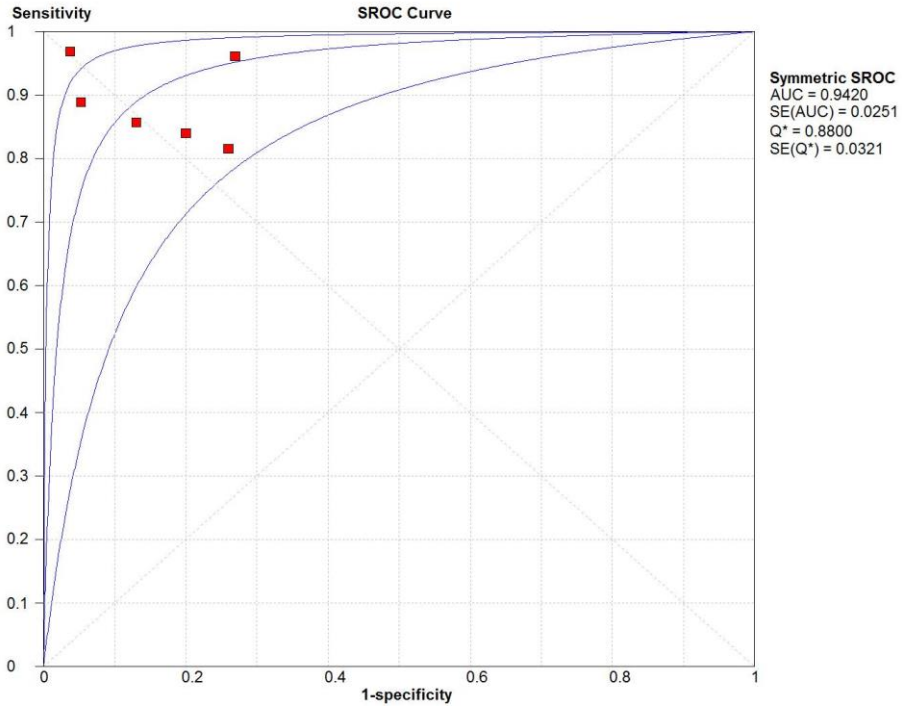

Figure 16 - Summary receiver operating characteristics (SROC) curve for exhaled VOC analysis in asthma diagnosis.

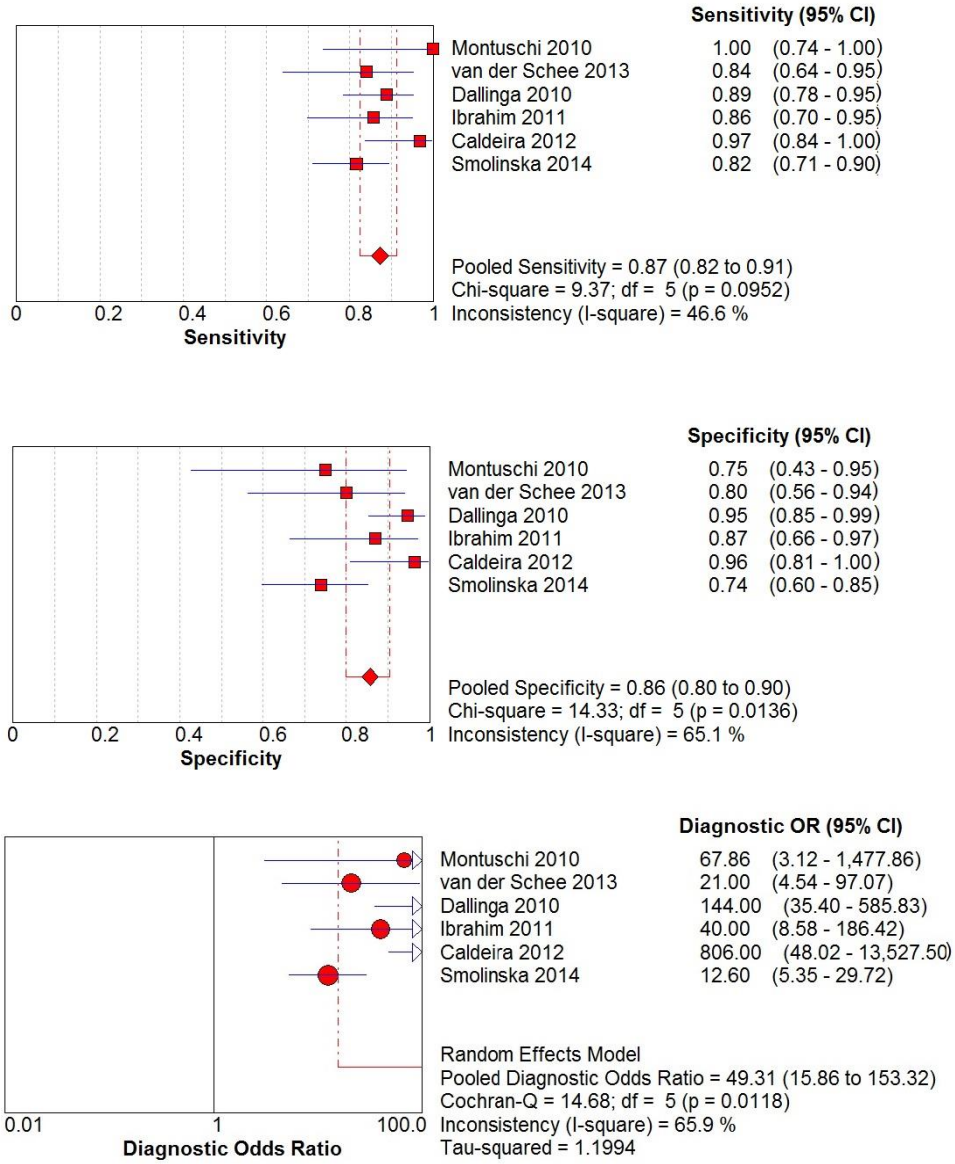

Figure 17 - Forest plot of the pooled sensitivity, specificity and diagnostic odds ratio of exhaled VOC analysis in asthma diagnosis.

5.4.1 Publication bias

To assess publication bias, the Begg's funnel plot and the Egger's test were used (Figure 18). Both the funnel plot and the Egger's test p-value (p=0.100) suggested no publication bias, although this observation is limited by the reduced number of studies included in the meta-analysis.

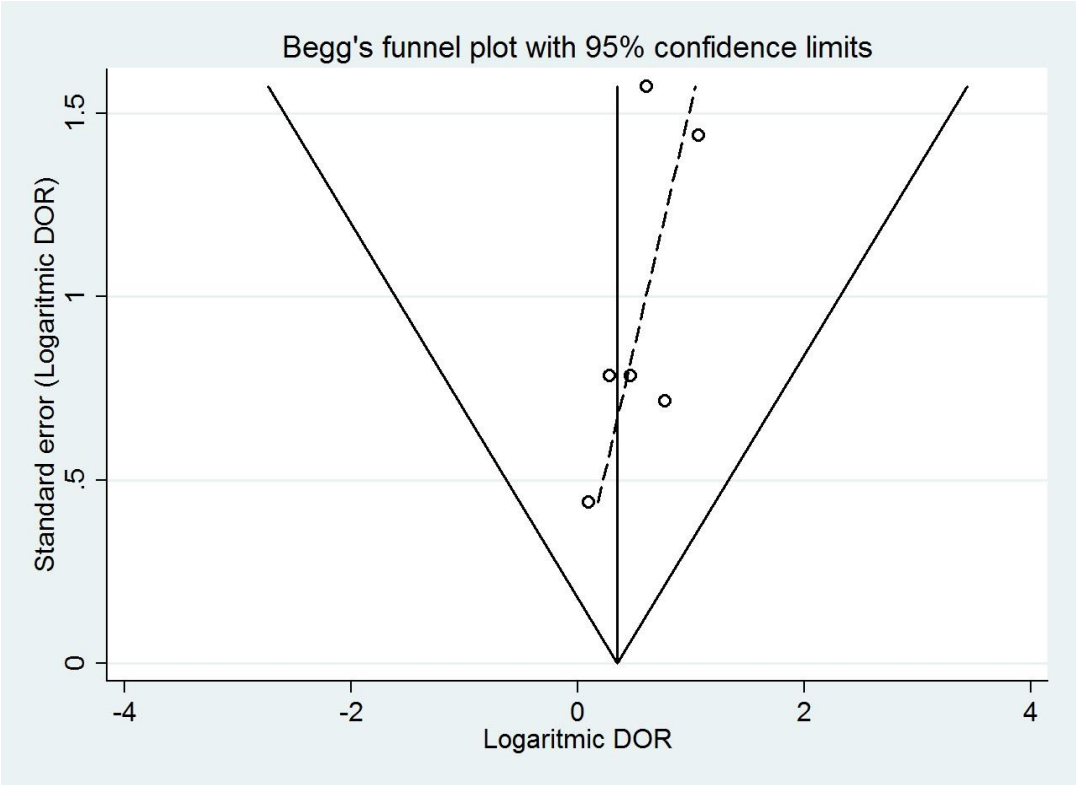

Figure 18 - Begg's funnel plot for evaluation of publication bias in the selected studies. The funnel graph plots the log of the diagnostic odds ratio (DOR) against the standard error of the log of the DOR (an indicator of sample size). The Egger test for publication bias was not statistically significant (p = 0.100), suggesting that there was no significant publication bias associated with the sample size. The dashed line represents Egger's test regression.

5.4.2 Diagnostic threshold and heterogeneity

To evaluate the diagnostic threshold, the Spearman's correlation coefficient between sensitivity and 1-specificity was calculated. The correlation coefficient was -0.429 (p=0.397), suggesting that there was no heterogeneity from the threshold effect. Moreover, heterogeneity was also explored by meta-regression to assess the contribution by sample size, type of methodology used and the country were the study was performed. However, evidence of heterogeneity was not found for none of the aforementioned characteristics (p>0.05).

5.5 Diagnostic accuracy of eNose-based exhaled VOC analysis for paediatric asthma (study IV)

Diagnostic accuracy measurements showed AUC values of 0.81 for both asthma diagnosis and persistent asthma diagnosis ($p<0.001$ in both cases), and achieved accuracy, sensitivity and specificity values ranging from 68.6 to 93.1%. Interestingly, although specificity values were generally lower, accuracy, sensitivity and AUC parameter values obtained from exhaled VOC analysis surpassed those from spirometry with bronchodilation in all cases. On the other hand, neither spirometry with bronchodilation or exhaled VOC analysis were useful in diagnosing intermittent asthma (AUC = 0.50 and 0.56, p=1.000 and p=0.457, respectively). Table 15 summarizes the paediatric asthma diagnostic accuracy parameters measured for both exhaled VOC analysis and spirometry with bronchodilation challenge.

Classification estimates showed a tendency to include individuals with asthma (OR=18.68; 95%CI: 4.50 to 77.24) and with a persistent disease (OR=29.50; 95%CI: 5.92 to 146.50) in cluster B (Figure 19).

Table 15 - Diagnostic accuracy parameters of the developed hierarchical model based on EBC VOC analysis through eNose in comparison with spirometry with bronchodilation.

	Method	Accuracy (%)	Sensitivity (%)	Specificity (%)	AUC (CI 95%)	*p*
Asthma diagnosis	BD	61.3	44.4	100	0.72 (0.60 - 0.84)	**0.005**
	eNose	79.7	77.8	84.2	0.81 (0.69 - 0.93)	**<0.001**
Intermittent asthma	BD	59.4	31.3	68.8	0.50 (0.34 - 0.67)	1.000
	eNose	40.6	50	37.5	0.56 (0.40 - 0.73)	0.457
Persistent asthma	BD	70.3	51.7	85.7	0.69 (0.55 - 0.82)	**0.010**
	eNose	79.7	93.1	68.6	0.81 (0.70 - 0.92)	**<0.001**

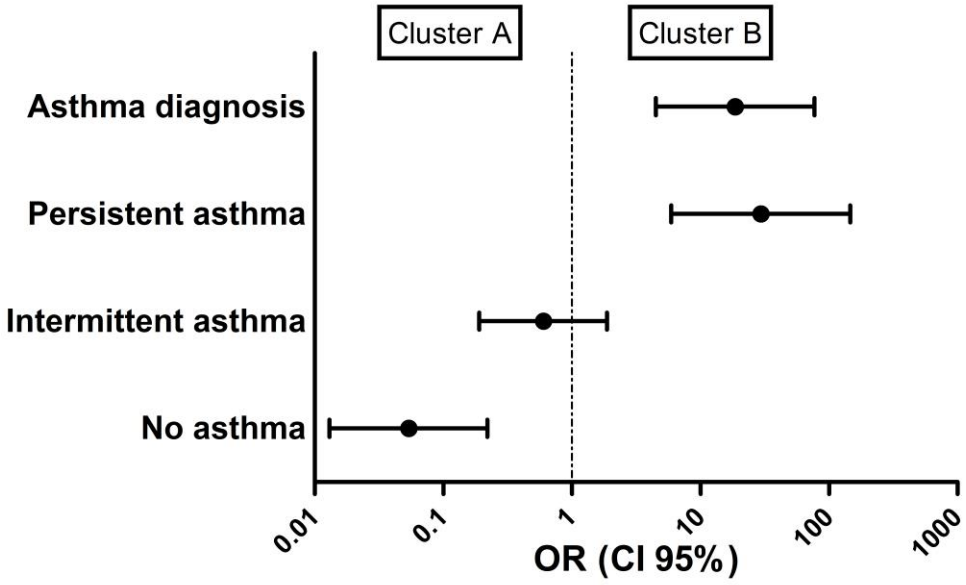

Figure 19 - Classification rates estimates between the two clusters of the hierarchical model.

6 Discussion

6.1 Methodologic considerations

Concerning the analysis of environmental determinants in classrooms and swimming pools (studies I and II), the main limitation resided in the cross-sectional design, which did not allow causal relationships to be established as a longitudinal study would. Specifically refering to the analysis of microbial determinants in paediatric asthma (study I), our observations considered only school-time exposure. While children spend most of their day at school, inside the classroom, they are still under exposure to microbiological agents in other environments when they sleep, or on the playground, during recess, therefore the complete exposome should be considered in future studies. Also, despite the fact that children in Portugal usually stay in the same classroom through primary school, this may not be true in other countries, resulting in more diverse exposure environments. Additionally, sensitization to one or more allergens does not necessarily imply a clinically relevant allergic status. Nevertheless, it was still possible to observe tendencies on how the prevalence of allergy and asthma in schoolchildren was affected by the exposure to indoor air bacteria and fungi in classrooms. Regarding the study focused on swimming pool exposure (study II), another limitation may be inherent to the question used to allocate participants to each group of swimming exposure, since although being "yes or no" questions, they are not exempt of reporting bias. Moreover, no data on number of hours of training per week has been considered for cumulative exposure assessment. However, considering the age range of participants, rarely should the practice represent more than 2 hours per week of active swimming. We also cannot exclude reverse causation bias as the number of children with otitis was lower in past swimmers.

Nevertheless, both studies (I and II) have several strengths. The substantial number of participants and classrooms involved provided good statistical power to the results, and the four distinct definitions of asthma may have prevented bias associated with the inclusion or exclusion of asymptomatic individuals. While concerning the microbial determinants (study I), diversity scores were calculated based on a large set of fungal species (24 in total), which enhanced the reliability of the obtained outcomes. On the other hand, the swimming pool exposure survey (study II) was community based, not affected by a swimming pool recruitment strategy bias, as children were recruited from 20 different schools. Moreover, the exclusion of sedentarism-based bias, coupled to the high number of

participants per group, as well as the extensive workup evaluation, particularly for assessing the autonomic nervous system and airway physiology, increased the robustness of the findings. Lastly, pupillometry is a sensible method that allows the detection of subtle changes in the oculosympathetic pathways, as previously demonstrated by Yoo *et al.* (2017) with the Horner's eye syndrome (Yoo *et al.*, 2017). These traits allowed the detection of asymptomatic alterations in parasympathetic tonus of children swimmers.

Regarding the systematic review (study III), the sensitivity and specificity values were retrieved from studies using not only different analysis methods but also different algorithms for VOC profiling. Therefore, despite how much promising the meta-analysis results may seem to be, the standardization of volatilome analysis should be conducted. In common with all meta–analyses, this systematic review may have included studies in which the characteristics of the subjects were too dissimilar for comparison or the spectrum of included patients may have not been completely representative. Nevertheless, this was considered and reported on the quality rating of the included studies, with consideration of eventual publication bias (Attachment I). Secondly, included studies used different methodologies for assessing exhaled VOCs (mostly eNose and GC-MS) and, more importantly, used distinct VOC profiles for diagnosing individuals with asthma, making it impossible to further investigate how the diagnostic potential correlates with these characteristics due to the reduced number of trials available.

There were also some limitations associated with the breathomics population recruitment design (study IV) such as the population size, since the total required population of 84 participants to surpass the 2.90 positive likelihood ratio reported for spirometry with bronchodilation was not achieved within its 95% confidence interval. Nevertheless, despite falling slightly short on individuals without asthma, the study managed to surpass the 42 mark for minimum individuals with asthma to be included, still reaching high sensitivity values. Another limitation may be associated with not separating individuals under different doses of inhaled corticosteroid therapy, which may have generated different OR values for the actual cluster according to the extent of airway inflammation. However, since the model was built blindly to the reference standard, the number of obtainable clusters would not change.

Despite the aforementioned limitations, the breathomics study has several important traits. Although several studies focused on asthma diagnosis through eNose technologies have already been published (Rufo *et al.*, 2016), most of these studies had either small sample sizes, biased analysis associated with the study design or statistical methods,

leading to methodologies inappropriate for real clinical application. In the present survey, recruitment was performed in two different settings, an outpatient allergy clinic and a football team training session, which hamper bias associated with the exclusive inclusion of individuals with allergic or respiratory conditions. Also, despite the fact that only football players were recruited during the training sessions, it was the variable that had the least influence on the model, thus showing that neither the recruitment setting or the participants' exercise activities have interfered with asthma diagnosis results. Stability and internal validation methods were adopted prior to model construction to assess clustering tendency. Moreover, although an exploratory analysis was performed, through PCA, the model itself was constructed blindly to the diagnostic standards (the 32 eNose sensor resistance values were the only variables used to create the model), thus reducing possible bias associated with pre-labelled group comparisons. The STARD guidelines for reporting diagnostic accuracy were followed throughout the study, reducing eventual reporting bias (Bossuyt *et al*., 2015). The reference standard results for asthma diagnosis were provided by physician's evaluation, according to standardized guidelines (Global Initiative for Asthma, 2017). Finally, breathomics were performed by eNose on EBC samples, which allowed the storage and freezing of samples, a considerably inexpensive 10 second per sample analysis, and the possibility of rapidly discriminating individuals with asthma, even in a hard-to-diagnose paediatric population.

6.2 Environmental determinants in paediatric asthma

6.2.1 The influence of microbial diversity exposure in classrooms (study I)

Although the findings showed a clear tendency for decreased allergic sensitization with a more diverse exposure to fungal species, similar tendencies were not observed for asthma. Correspondingly, the increased risk of allergic sensitization that was found to be associated with higher levels of endotoxin or *Penicillium spp* was not detected for asthma.

The enhanced risk of allergic sensitization with higher endotoxin exposure had already been expressed in the PASTURE study (Karvonen *et al*., 2012). These results need to be interpreted with caution, however, since children in the present study were considerably older and may have their immune systems further developed, thus the impact of exposure to endotoxin concentrations in this age group may be substantially different

than when exposition occurs in pre and post-natal periods of life. This may also justify why, unlike in previous studies (Braun-Fahrlander et al., 2002, Thorne et al., 2005, Ege et al., 2011, Mendy et al., 2011, Karvonen et al., 2012), exposure to environmental endotoxins had no significant associations with asthma prevalence. A further matured immune system may also be responsible for the distinct results regarding exhaled NO associations with microbiological parameters between the present work and the study performed by Casas *et al*. (2013), which showed that exhaled NO at school age was lower in children exposed to endotoxins during the first 2 to 3 months of life. Therefore, these results support that allergy and asthma have different responses to the same environmental determinants, and that a diverse exposure to these microbial agents appear to contribute to a preventive immunomodulation in children, inducing tolerance to allergens trough both adaptive and innate immune mechanisms (Cavaleiro Rufo *et al*., 2017b).

While no particular fungal species were associated with any of the 4 definitions of paediatric asthma, the risk for allergic sensitization was significantly lower in classrooms with higher concentrations of some *Aspergillus* species, along with *Chaetomium* and *Rhizopus* species, thus supporting the results reported by Ege *et al*. (2011) in the framework of the GABRIELA study . On the other hand, classrooms with higher concentrations of *Penicillium spp* showed a significantly higher prevalence of allergic sensitization among children, which may correspond to similarly high cases of asthma onset later in life, thus corroborating the results obtained in a meta-analysis by Sharpe *et al*. (2015), where *Penicillium* species were found in significantly higher concentrations in the homes of individuals with asthma. It is possible that the higher sensitization is associated with the lower diversity scores in the respective classrooms, as high concentrations of *Penicillium spp* may extensively reduce the sustainability of other fungi species. In contrast, classrooms with a higher fungal diversity and lower concentrations of *Penicillium spp* were shown to be negatively associated with the prevalence of allergic sensitization, supporting the results from previous studies (Dannemiller et al., 2014, Behbod et al., 2015). Despite having a clear impact on sensitization, fungal diversity was not a risk factor for allergic rhinitis and atopic dermatitis, suggesting that exposure to microbiological agents may differently influence allergic diseases.

Nevertheless, increased fungal diversity showed a tendency to be associated with a higher risk for lung function diagnosed asthma, but not for the other definitions. Children with lung function diagnosed asthma are generally in an uncontrolled or severe state of the disease. Therefore, a possible interpretation for these results may be hypothesized through

the fact that these children may already had a compromised immunomodulation and poor tolerance to fungal allergens, leading to an exacerbate state (with lung function alterations) of asthma when exposed to these environmental triggers. On the other hand, sensitization odds ratio was lower than the unity for all terciles and a significant tendency for a lower risk of allergic sensitization was observed in classrooms with higher fungal diversity, suggesting that exposure to environmental fungi has a completely different impact in asthma and allergy. Interestingly, when having all these results in mind, microbial diversity appears to be an environmental determinant primarily for allergic sensitization (either by promoting immunomodulation and prevention, or by leading to exacerbations when immune tolerance fails) and may only indirectly influence asthma prevalence through development of the allergic disease. Part of these results are in line with recent advances in the biodiversity and microbiome hypothesis (Fujimura and Lynch, 2015), supporting the importance of exposure to a diverse biome to prevent development of allergic sensitization and eventual development of allergic asthma in the paediatric population. Moreover, the results supporting the influence of classrooms' microbiota biodiversity on early immunomodulation are interestingly similar to the those found by Ruokolainen *et al.* (2015) in green areas around cities. On the other hand, the results failed to support the evidence of beneficial exposure to bacterial endotoxins as observed in other studies concerning the biodiversity hypothesis (Carlsten *et al*., 2011). However, these are not isolated results. For instance, a domestic endotoxin exposure study in Cyprus also found higher exposure to endotoxins to be associated with increased allergic sensitization (Nicolaou *et al*., 2006). It is possible that regional environmental and genotypic differences (for instance, Mediterranean versus Nordic regions) may have a role in this controversy. More region-wide studies are needed to further clarify the biodiversity hypothesis.

In conclusion, the exposure to microbial diversity in classrooms does not directly influence asthma prevalence in children, but appears to be a strong environmental determinant of allergic sensitization in the paediatric population, mediating immunomodulation and tolerance, which when not properly conducted, may result in development of the allergic disease, eventually leading to exacerbations in children with asthma. In face of these evidences, understanding the factors that influence microbial diversity in the school environment may lead to public health recommendations for reducing the development of allergic sensitization or prevent symptomatic exacerbations of asthma in the future.

6.2.2 Swimming pool exposure and increased airway reactivity to a beta-2 agonist (study II)

In this study, swimming pool attendance was associated with autonomic changes and airway alterations even in non-elite swimmers. Firstly, pupillometry showed that swimming pool exposure in school-aged children is associated with parasympathetic dysautonomia. Secondly, a higher volume of airway reversibility in response to an inhaled beta-2 agonist, suggestive of increased baseline airway smooth muscle constriction, was also observed in children that frequently attend swimming pools. Thirdly, these subtle changes appear to be reversible with swimming practice cessation, as no evidence of parasympathetic perturbances or airway constriction were found in children that used to swim in the past, even if they kept practicing any other type of sport.

Neurogenic inflammation has been suggested to contribute to the recognized higher prevalence of asthma in athletes training and competing in environments with a high airway irritation potential (Silva and Moreira, 2015). Transient receptor potential vanilloid 1 (TRPV1) is the centre of almost all neuronal inflammatory signalling pathways; this ion channel is often co-localized with sensory neuropeptides in the same axon of a primary neuron and its stimulation can lead to the release of these substances. It is expressed in primary sensory neurons, pulmonary smooth muscle cells, bronchial and tracheal epithelial cells and dendritic cells in the lung (Colsoul *et al*., 2009). Known physical activators of these channels include noxious temperature such as heat or cold, changes in membrane potential, mechanical or osmotic stress, and arachidonic acid metabolites (Banner *et al*., 2011). A recent study showed increased levels of substance P in sputum of competitive swimmers suggesting it may be the result of a compensatory response to the sympathetic stimulation promoted by intensive training, neurogenic inflammatory response to swim stress and/or a local airway chemosensory reflex to chlorine by-products exposure in swimmers (Ramalho *et al*., 2014). However, altered parasympathetic tonus in healthy swimmers has until this moment been almost exclusively associated with endurance training. Although the high training volumes may certainly influence the autonomic nervous function, this study results now show that parasympathetic dysfunction does occur in healthy children swimmers not undergoing endurance training. Our findings further extend this observation suggesting that autonomic changes may not only be caused by high training volumes, but also by environmental exposure, even in young children. The

significantly higher exhaled NO levels observed in swimmers, although subtle and within physiologic reference values, support the co-existence of airways inflammation in those children. Therefore, it is possible that swimming pool exposure may be responsible for airway constriction at two levels: directly, by inhalation of disinfection by-products which will damage airway epithelium and increase oxidative stress (Bernard *et al*., 2015); and indirectly, by causing parasympathetic dysautonomia which may lead to a reflex vasoconstriction of bronchial venules, reducing the size of the bronchial lumen and generating increased airways resistance (Giacco *et al*., 2015).

In the present study, the subtle changes associated with swimming pool attendance tended to disappear in children who discontinued swimming practice, since autonomic parameters, lung function and exhaled NO levels were not significantly different between past and non-swimmers. These results, coupled to the absence of significant correlations between the number of years in swimming practice and the airway reversibility or exhaled NO, suggests that swimming pool environment was the main responsible for the changes in airway inflammation biomarkers, which tend to disappear after ceasing the practice. In addition, parasympathetic activity also appears to be re-established after swimming cessation, since only sympathetic parameters seem to be significantly correlated with the number of years in swimming practice. Although this changes in sympathetic activity may be associated with more years of regular physical exercise (Sarmento *et al*., 2017), this cannot be concluded in the present study since only swimming practice was considered and children may have performed other physical activities at the same time period. Interestingly, these "reversibility" results support the hypothesis presented by Lomax (2016) regarding airway dysfunction in elite swimmers (Lomax, 2016). By systematically reviewing relevant publications, Lomax hypothesised that chlorine exposure in swimming pools coupled to endurance swimming exercises caused epithelial damage that could lead to several airway symptoms, including bronchoconstriction (Anderson and Kippelen, 2005), but airway epithelium was estimated to be replenished every 30 to 50 days in the absence of continued damage (Williams, 2012, Lomax, 2016). When under continuous exposure, the injury-repair process of the airways epithelium may lead to respiratory disorders and, eventually, airway hyperresponsiveness (Anderson and Kippelen, 2005, Bougault *et al*., 2009, Lomax, 2016). While children in the present study were certainly not submitted to endurance training, they were still exposed to chlorine-based disinfection by-products.

It is important to notice the several positive traits associated with swimming pool attendance. In line with other population-based studies (Goodman and Hays, 2008, Font-Ribera et al., 2009, Font-Ribera et al., 2011, Jacobs et al., 2012, Font-Ribera et al., 2014), the results showed that swimming pool attendance was not associated with increased of allergic sensitization in children, although a trend for a higher risk of asthma (functional criteria), atopic eczema and rhinitis, was observed in swimmers. Swimming practice was also not associated with a higher prevalence of allergic sensitization and no adverse effects on baseline lung function parameters were observed. In fact, this study shows that children with more years in swimming practice generally have improved baseline lung function, supporting several other studies in the last two decades (Doherty and Dimitriou, 1997, Lazovic-Popovic *et al*., 2016), including those focused in prepubertal children, such as the one published by Courteix *et al* in 1997 (Courteix *et al*., 1997). However, seldom has exercise-induced bronchoconstriction been scrutinized in these studies and, as observable in the present study, children that attend swimming pools have a significantly higher exhaled NO and reversibility of FEV_1. These results, coupled to the altered parasympathetic function in the current swimmers group suggests that exposure during indoor swimming may contribute to airways constriction independently of the training volume and asthma status.

Two important aspects of the present study need to be taken into consideration: first, the airway reversibility and eosinophilic airway inflammation observed in active swimmers group are non-pathological according to current guidelines (Dweik *et al*., 2011, Global Initiative for Asthma, 2017); and second, the measured parameters appear to normalize after the practice cessation, as observed with the children in the PS group, independently of continuing other sportive activities. While the physiological mechanisms of the association between altered autonomic function and environmental exposure have not been fully explained, there is evidence showing that traffic-related air pollution may be responsible for disturbances of the autonomic system (Schwartz *et al*., 2005). Baja *et al.* (2013), using structural equation models, also observed that traffic pollution may decrease parasympathetic tone among diabetic elderly (Baja *et al*., 2013). Therefore, we may assume the observed autonomic changes could be associated with indoor swimming pool attendance in susceptible individuals, such as schoolchildren.

Overall, while microbial exposure was only shown to indirectly influence asthma onset or exacerbations through allergic sensitization, swimming pool attendance appears to directly influence subtle changes associated with asthma like symptoms (including airway

smooth muscle constriction) in school-aged children. Although these changes are yet to reach pathological levels, they somehow resemble the large-scale changes associated with exercise-induced asthma in adults. Hence, exposure to swimming pool disinfectants should be considered as an environmental determinant for paediatric asthma.

6.3 Exhaled VOC analysis in asthma diagnosis

6.3.1 Accuracy of exhaled VOC biomarkers in asthma diagnosis (study III)

The systematic review and meta-analysis showed that: firstly, exhaled VOC profiles have high sensitivity and specificity values for asthma diagnosis; secondly, individuals with asthma had 6 times higher chance of being diagnosed through exhaled VOC profiles than healthy controls; and thirdly, diagnosis odds ratios and AUC values were respectively 49.3 and 0.94. Taken together, these observations suggest that exhaled VOC profile analysis may be an accurate test for asthma diagnosis. Nevertheless, these results should be interpreted with caution since the sensitivity and specificity values were retrieved from studies using not only different analysis methods but also different algorithms for VOC profiling. Therefore, despite how much promising the meta-analysis results may seem to be, there is still much to be done before introducing VOC biomarkers in a real clinical context.

These findings are particularly important when compared with the sensitivity and specificity values of the currently used clinical tools in asthma diagnosis. For instance, the pooled sensitivity values of VOC profiles (87%) were higher than those obtained by spirometry considering both FEV_1<80% and <90% (29% and 35%, respectively) (Smith *et al*., 2004, Schneider *et al*., 2009). Bronchodilator reversibility >12% also showed a poor sensitivity value (36%) when compared with VOC profiles (Tse *et al*., 2013). On the other hand, the GINA self-reported symptom questionnaires showed highly skewed sensitivity/specificity ratios (10.9 for ≥1 reported symptoms and 0.2 for ≥5 reported symptoms) while VOC profiles presented a ratio approximated to 1.0 (Lim *et al*., 2014).

The systematic review also revealed that exhaled VOC analysis may be used for differentiating diseases that share symptoms with asthma, and since comorbidity is frequent in children, this is an attractive trait for paediatric asthma diagnosis. However, most of the reviewed studies were focused on comorbidities associated with elderly

subjects. Such is the case of COPD, which is often misdiagnosed as asthma in older individuals, particularly those who were significantly exposed to tobacco earlier in life. In a study conducted in the Netherlands, by Fens and co-workers (2009), the authors aimed to correctly distinguish a population comprised by 30 subjects with COPD, 20 subjects with asthma and 40 healthy controls, analysing their exhaled breath by eNose. The results showed that the breath-prints of individuals with asthma were significantly different from those with COPD or with no respiratory diseases (controls) with an accuracy of respectively 96 and 95%. The external validation of the mentioned results was demonstrated in a later study by Fens *et al*. (2011) were they showed that the accuracy for discriminating asthma from COPD was not confounded by "current smoking" status. However, these results were based on training sets, which required the reference standard to be known previously to the index test, thus being susceptible to diagnostic reporting bias according to STARD (Bossuyt *et al*., 2015). In addition, different results were obtained by Schivo *et al*. (2013), who after analysing exhaled VOC profiles through GC-DMS, observed that it was not possible to distinguish between COPD and asthma. Nevertheless, the small size of the sampled population (13 individuals with asthma and 5 with COPD) was pointed by the authors as the main reason behind these results.

Contrasting with COPD, gastro-oesophageal reflux disease symptoms are not uncommon in children (Dent *et al*., 2005). Timms and co-workers (2012) performed a study aiming to distinguish patients suffering from common obstructive lung diseases (including asthma) with concomitant gastro-oesophageal reflux disease from those without gastro-oesophageal reflux disease, using eNose technology. The population included 20 individuals with asthma and the results showed that the breath-prints from individuals with gastro-oesophageal reflux disease were highly distinguishable from those without reflux, in the asthma population.

Unfortunately, there was limited research data concerning exhaled VOCs analysis to distinguish different phenotypes of asthma, which would enhance the potential of the method to assist the physician in properly prescribing corticosteroid treatment for paediatric asthma. Nevertheless, several studies showed that is possible to differentiate asthma severity using exhaled VOCs assessment (Olopade et al., 1997, Paredi et al., 2000, Delfino et al., 2003, Robroeks et al., 2013). This has also been shown using eNose technology, as demonstrated by the promising results from the study performed by van der Schee *et al*. (2013), where exhaled VOC profiles were able to predict steroid

responsiveness in patients with asthma with greater accuracy than exhaled NO and sputum eosinophils count.

Concluding, the systematic review showed that exhaled VOC analysis may be used for both asthma diagnosis and severity monitoring. There is, however, a need for more studies contemplating proper internal and external validation procedures. In addition, further research on exhaled VOCs released from EBC samples needs to be conducted, preferably undergoing standardisable proceedings to allow reliable comparisons between studies.

6.3.2 Electronic nose analysis of VOCs in EBC is able to identify children with asthma and those under corticosteroid therapy (study IV)

The developed hierarchical model based on eNose VOC analysis of EBC samples was able to distinguish individuals with a medical diagnosis of paediatric asthma. In addition, persistent asthma patients that were under the need of inhaled corticosteroid therapy were significantly discernible with high accuracy, sensitivity and AUC values. These results considerably surpassed those from spirometry with bronchodilation challenge in asthma diagnosis. Therefore, exhaled VOC analysis, even when conducted through EBC samples, may be used as a handy complementary diagnostic methodology to assist the physician's decision in administering corticosteroid therapy for paediatric patients with asthma.

To our knowledge, this is the first study showing a methodology cable of distinguishing individuals with asthma through eNose EBC analysis. Overall, it is shown that breathprints in EBC provide similar AUC values to those shown in other studies using gas-phased exhaled breath and/or gas-chromatography methodologies for asthma diagnosis (Dallinga et al., 2010, Montuschi et al., 2010, Ibrahim et al., 2011, Caldeira et al., 2012, van der Schee et al., 2013, Smolinska et al., 2014). In fact, the measured VOC profiles allowed the inclusion of 93.1% individuals with persistent asthma and all of those with positive bronchodilation challenge in the same cluster of the developed model. Still, considering the high specificity of spirometry, both techniques could be used in combination to achieve a significantly reliable asthma diagnosis.

The fact that individuals with persistent asthma were significantly distinguishable from those with intermittent asthma may be associated with the different degree of airway inflammation at sample collection. This is supported by the odds ratio results, which showed a significant tendency for medical asthma diagnosis and persistent asthma to be

included in the same cluster. As expected, individuals without asthma were significantly associated with the opposite cluster of the model, thus providing a clear opposing tendency for asthma diagnosis between clusters.

The inflammatory process results in the excretion of specific metabolites through exhaled air, providing different volatile biomarkers in the condensate samples (Kharitonov and Barnes, 2001). These specific VOCs cannot be identified by eNose, as a more metabolomics-specialized method is required, such as GC-MS (Van Berkel et al., 2008, Ibrahim et al., 2011, Caldeira et al., 2012, Couto et al., 2017). However, the GC-MS methodology takes a substantial amount of time to analyse a sample, when compared to the 10 second eNose sampling time, and requires a properly equipped laboratory meeting safety conditions to hold gas tanks. Moreover, instrumentation and analysis costs are considerably higher. Altogether, it would be difficult to apply GC-MS analysis in clinical settings when compared to a portable and relatively independent eNose system.

Interestingly, participants with intermittent asthma were successfully discernible in the model, which again supports the discriminant potential of inflammation-originated VOCs. Although individuals with persistent asthma were included in the same group at the present study, Dragonieri *et al.* (2007) showed the breathprints of individuals with mild asthma are also discernible from those with severe asthma, even though a cross-validation value of only 65% was reported (Dragonieri *et al.*, 2007). Therefore, although more studies with larger populations are needed, it is possible that exhaled VOC analysis may be able to further classify asthma severity according to airway inflammation degree. These results suggest that eNose exhaled VOC analysis would be particularly useful to identify individuals in need of corticosteroid therapy, thus assisting physician's in reducing mistreatment or overtreatment of asthma in the paediatric population (Chung, 2016).

Although the developed hierarchical model was already blindly built to the diagnostic standard results, an external validation of the model, tested on a new sample of individuals, should be performed to complete the STARD recommendations and to start implementing eNose VOC analysis on EBC samples for rapid and complementary asthma diagnosis in clinical settings.

6.4 Implications for practice and future research

The results presented on this thesis support the need to further study environmental determinants of paediatric asthma in order to improve disease prevention. Although classroom and swimming pool exposure in children has been shown to influence (either directly or indirectly) asthma development or exacerbation, fully understanding the mechanisms behind the onset trigger would help to identify important prospects for eventual preventive therapeutics and behaviours. Moreover, epigenetics may be influencing the impact of environmental determinants, and it needs to be taken into account in future studies. In fact, this is already being performed by the Health and Environment-wide Associations based on Large population Surveys (HEALS) project, using a cohort of tweens to comprehend the impact of epigenetics on environmental exposure susceptibility in children.

In relation to exhaled VOC analysis, this thesis showed interesting results provided by an innovative methodology capable of accurately diagnosing paediatric asthma and distinguishing individuals under inhaled corticosteroid treatment. The sample collection and laboratory methodology presented in this work were designed while having in mind a possible implementation on a large-scaled clinical setting. Nevertheless, standardization procedures and external validation of the developed hierarchical model still need to be performed in order to successfully implement exhaled VOC analysis through eNose in a real clinical context.

Overall, the present thesis is expected to contribute to the prevention and treatment of paediatric asthma, hopefully reducing its astonishing prevalence, morbidity and severity in a near future.

7 Conclusions

In the present thesis, the highly prevalent paediatric asthma has been studied in terms of immunomodulatory environmental determinants and in means for correctly diagnosing (and consequently treating) this challenging disease. Based on the results obtained throughout the performed studies, the following conclusions were drawn:

1. The exposure to microbial diversity in classrooms does not directly influence asthma prevalence in children, but may influence allergic sensitization in the paediatric population by mediating immunomodulation and allergen tolerance. Consequently, when this tolerance is not properly achieved, allergic disease may further develop with microbial exposure, eventually leading to exacerbations in children with asthma.

2. Swimming pool attendance is associated with autonomic changes even in non-elite swimmers, such as children. Continued swimming pool exposure in school-aged children may cause parasympathetic dysautonomia and increased response to inhaled beta-2 agonists, consequently resulting in increased baseline airway smooth muscle constriction. These subtle changes appear to be reversible with swimming practice cessation.

3. Exhaled VOCs have high sensitivity, specificity and AUC values for asthma diagnosis, and may assist the physician in determining the appropriate treatment for paediatric-aged individuals. However, there are still various constraints associated with standardization and validation of the collection and analysis procedures.

4. Electronic nose analysis of EBC is able to distinguish individuals with asthma from those without asthma, and to identify individuals in need of corticosteroid therapy, even in a paediatric population, showing higher overall accuracy, sensitivity and AUC values when compared to spirometry with bronchodilation challenge.

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References

AHLUWALIA, S. K. & MATSUI, E. C. 2011. The indoor environment and its effects on childhood asthma. *Curr Opin Allergy Clin Immunol,* 11**,** 137-43.

ALI, Z., O'HARE, W. & THEAKER, B. 2003. Detection of bacterial contaminated milk by means of a quartz crystal microbalance based electronic nose. *Journal of Thermal Analysis and Calorimetry,* 71**,** 155-161.

ANDERSON, S. D. 2008. Provocative challenges to help diagnose and monitor asthma: exercise, methacholine, adenosine, and mannitol. *Curr Opin Pulm Med,* 14**,** 39-45.

ANDERSON, S. D. & KIPPELEN, P. 2005. Exercise-induced bronchoconstriction: pathogenesis. *Curr Allergy Asthma Rep,* 5**,** 116-22.

ANDERSSON, M., HEDMAN, L., NORDBERG, G., FORSBERG, B., ERIKSSON, K. & RONMARK, E. 2015. Swimming pool attendance is related to asthma among atopic school children: a population-based study. *Environ Health,* 14**,** 37.

ARBES, S. J., GERGEN, P. J., VAUGHN, B. & ZELDIN, D. C. 2007. Asthma Cases Attributable to Atopy: Results from the Third National Health and Nutrition Examination Survey. *The Journal of allergy and clinical immunology,* 120**,** 1139-1145.

ASHER, I. & PEARCE, N. 2014. Global burden of asthma among children. *Int J Tuberc Lung Dis,* 18**,** 1269-78.

ASHER, M. I., KEIL, U., ANDERSON, H. R., BEASLEY, R., CRANE, J., MARTINEZ, F., MITCHELL, E. A., PEARCE, N., SIBBALD, B., STEWART, A. W. & ET AL. 1995. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. *Eur Respir J,* 8**,** 483-91.

BAENA-CAGNANI, C. E. & BADELLINO, H. A. 2011. Diagnosis of allergy and asthma in childhood. *Curr Allergy Asthma Rep,* 11**,** 71-7.

BAHADORI, K., DOYLE-WATERS, M. M., MARRA, C., LYND, L., ALASALY, K., SWISTON, J. & FITZGERALD, J. M. 2009. Economic burden of asthma: a systematic review. *BMC Pulm Med,* 9**,** 24.

BAJA, E. S., SCHWARTZ, J. D., COULL, B. A., WELLENIUS, G. A., VOKONAS, P. S. & SUH, H. H. 2013. Structural equation modeling of parasympathetic and sympathetic response to traffic air pollution in a repeated measures study. *Environmental Health,* 12**,** 81-81.

BANERJEE, A. & DAVE, R. N. Validating clusters using the Hopkins statistic. 2004 IEEE International Conference on Fuzzy Systems (IEEE Cat. No.04CH37542), 25-29 July 2004 2004. 149-153 vol.1.

BANNER, K., IGNEY, F. & POLL, C. 2011. TRP channels: emerging targets for respiratory disease. *Pharmacol Ther,* 130**,** 371-84.

BARATLOO, A., HOSSEINI, M., NEGIDA, A. & EL ASHAL, G. 2015. Part 1: Simple Definition and Calculation of Accuracy, Sensitivity and Specificity. *Emergency,* 3**,** 48-49.

BEHBOD, B., SORDILLO, J. E., HOFFMAN, E. B., DATTA, S., WEBB, T. E., KWAN, D. L., KAMEL, J. A., MUILENBERG, M. L., SCOTT, J. A., CHEW, G. L., PLATTS-MILLS, T. A., SCHWARTZ, J., COULL, B., BURGE, H. & GOLD, D. R. 2015. Asthma and allergy development: contrasting influences of yeasts and other fungal exposures. *Clin Exp Allergy,* 45**,** 154-63.

BELDA, J., RICART, S., CASAN, P., GINER, J., BELLIDO-CASADO, J., TORREJON, M., MARGARIT, G. & DROBNIC, F. 2008. Airway inflammation in the elite athlete and type of sport. *Br J Sports Med,* 42**,** 244-8; discussion 248-9.

BERGERON, C., BOULET, L. P., PAGE, N., LAVIOLETTE, M., ZIMMERMANN, N., ROTHENBERG, M. E. & HAMID, Q. 2007. Influence of cigarette smoke on the arginine pathway in asthmatic airways: increased expression of arginase I. *J Allergy Clin Immunol,* 119**,** 391-7.

BERNARD, A., CARBONNELLE, S., DUMONT, X. & NICKMILDER, M. 2007. Infant swimming practice, pulmonary epithelium integrity, and the risk of allergic and respiratory diseases later in childhood. *Pediatrics,* 119**,** 1095-103.

BERNARD, A., NICKMILDER, M. & DUMONT, X. 2015. Chlorinated pool attendance, airway epithelium defects and the risks of allergic diseases in adolescents: Interrelationships revealed by circulating biomarkers. *Environ Res,* 140**,** 119-26.

BERNARD, A., NICKMILDER, M., VOISIN, C. & SARDELLA, A. 2009. Impact of chlorinated swimming pool attendance on the respiratory health of adolescents. *Pediatrics,* 124**,** 1110-8.

BERNSTEIN, I. L., LI, J. T., BERNSTEIN, D. I., HAMILTON, R., SPECTOR, S. L., TAN, R., SICHERER, S., GOLDEN, D. B., KHAN, D. A., NICKLAS, R. A., PORTNOY, J. M., BLESSING-MOORE, J., COX, L., LANG, D. M., OPPENHEIMER, J., RANDOLPH, C. C., SCHULLER, D. E., TILLES, S. A., WALLACE, D. V., LEVETIN, E. & WEBER, R. 2008. Allergy diagnostic testing: an updated practice parameter. *Ann Allergy Asthma Immunol,* 100**,** S1-148.

BICKEL, S., POPLER, J., LESNICK, B. & EID, N. 2014. Impulse oscillometry: interpretation and practical applications. *Chest,* 146**,** 841-847.

BOOTS, A. W., BOS, L. D., VAN DER SCHEE, M. P., VAN SCHOOTEN, F. J. & STERK, P. J. 2015. Exhaled Molecular Fingerprinting in Diagnosis and Monitoring: Validating Volatile Promises. *Trends Mol Med,* 21**,** 633-44.

BORENSTEIN, M., HEDGES, L. V., HIGGINS, J. P. T. & ROTHSTEIN, H. R. 2009. *Introduction to Meta-Analysis.,* Oxford, Wiley.

BOS, L. D., STERK, P. J. & FOWLER, S. J. 2016. Breathomics in the setting of asthma and chronic obstructive pulmonary disease. *J Allergy Clin Immunol,* 138**,** 970-976.

BOSSUYT, P. M., REITSMA, J. B., BRUNS, D. E., GATSONIS, C. A., GLASZIOU, P. P., IRWIG, L., LIJMER, J. G., MOHER, D., RENNIE, D., DE VET, H. C. W., KRESSEL, H. Y., RIFAI, N., GOLUB, R. M., ALTMAN, D. G., HOOFT, L., KOREVAAR, D. A. & COHEN, J. F. 2015. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. *BMJ : British Medical Journal,* 351.

BOUGAULT, V., TURMEL, J., ST-LAURENT, J., BERTRAND, M. & BOULET, L. P. 2009. Asthma, airway inflammation and epithelial damage in swimmers and cold-air athletes. *Eur Respir J,* 33**,** 740-6.

BRAMAN, S. S. 2006. The global burden of asthma. *Chest,* 130**,** 4s-12s.

BRAUN-FAHRLANDER, C., RIEDLER, J., HERZ, U., EDER, W., WASER, M., GRIZE, L., MAISCH, S., CARR, D., GERLACH, F., BUFE, A., LAUENER, R. P., SCHIERL, R., RENZ, H., NOWAK, D. & VON MUTIUS, E. 2002. Environmental

exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med,* 347**,** 869-77.

BURBANK, A. J., SOOD, A. K., KESIC, M. J., PEDEN, D. B. & HERNANDEZ, M. L. 2017. Environmental determinants of allergy and asthma in early life. *J Allergy Clin Immunol,* 140**,** 1-12.

BURYAKOV, I. A., KRYLOV, E. V., NAZAROV, E. G. & RASULEV, U. K. 1993. A new method of separation of multi-atomic ions by mobility at atmospheric pressure using a high-frequency amplitude-asymmetric strong electric field. *International Journal of Mass Spectrometry and Ion Processes,* 128**,** 143-148.

BUSZEWSKI, B., KESY, M., LIGOR, T. & AMANN, A. 2007. Human exhaled air analytics: biomarkers of diseases. *Biomed Chromatogr,* 21**,** 553-66.

CALDEIRA, M., BARROS, A. S., BILELO, M. J., PARADA, A., CAMARA, J. S. & ROCHA, S. M. 2011. Profiling allergic asthma volatile metabolic patterns using a headspace-solid phase microextraction/gas chromatography based methodology. *J Chromatogr A,* 1218**,** 3771-80.

CALDEIRA, M., PERESTRELO, R., BARROS, A. S., BILELO, M. J., MORETE, A., CAMARA, J. S. & ROCHA, S. M. 2012. Allergic asthma exhaled breath metabolome: a challenge for comprehensive two-dimensional gas chromatography. *J Chromatogr A,* 1254**,** 87-97.

CARLSTEN, C., FERGUSON, A., DIMICH-WARD, H., CHAN, H., DYBUNCIO, A., ROUSSEAU, R., BECKER, A. & CHAN-YEUNG, M. 2011. Association between endotoxin and mite allergen exposure with asthma and specific sensitization at age 7 in high-risk children. *Pediatr Allergy Immunol,* 22**,** 320-6.

CARRARO, S., GIORDANO, G., RENIERO, F., CARPI, D., STOCCHERO, M., STERK, P. J. & BARALDI, E. 2013. Asthma severity in childhood and metabolomic profiling of breath condensate. *Allergy,* 68**,** 110-117.

CARRARO, S., REZZI, S., RENIERO, F., HEBERGER, K., GIORDANO, G., ZANCONATO, S., GUILLOU, C. & BARALDI, E. 2007. Metabolomics applied to exhaled breath condensate in childhood asthma. *Am J Respir Crit Care Med,* 175**,** 986-90.

CARTER, J. V., PAN, J., RAI, S. N. & GALANDIUK, S. 2016. ROC-ing along: Evaluation and interpretation of receiver operating characteristic curves. *Surgery,* 159**,** 1638-1645.

CASAS, L., TISCHER, C., WOUTERS, I. M., TORRENT, M., GEHRING, U., GARCIA-ESTEBAN, R., THIERING, E., POSTMA, D. S., DE JONGSTE, J., SMIT, H. A., BORRAS-SANTOS, A., ZOCK, J. P., HYVARINEN, A., HEINRICH, J. & SUNYER, J. 2013. Early life microbial exposure and fractional exhaled nitric oxide in school-age children: a prospective birth cohort study. *Environ Health,* 12**,** 12-103.

CAVALEIRO RUFO, J., MADUREIRA, J., PACIENCIA, I., AGUIAR, L., PEREIRA, C., SILVA, D., PADRAO, P., MOREIRA, P., DELGADO, L., ANNESI-MAESANO, I., OLIVEIRA FERNANDES, E., TEIXEIRA, J. P. & MOREIRA, A. 2017a. Indoor fungal diversity in primary schools may differently influence allergic sensitization and asthma in children. *Pediatr Allergy Immunol,* 28**,** 332-339.

CAVALEIRO RUFO, J., MADUREIRA, J., PACIENCIA, I., SLEZAKOVA, K., PEREIRA MDO, C., AGUIAR, L., TEIXEIRA, J. P., MOREIRA, A. & OLIVEIRA FERNANDES, E. 2016a. Children exposure to indoor ultrafine particles in urban and rural school environments. *Environ Sci Pollut Res Int,* 23**,** 13877-85.

CAVALEIRO RUFO, J., MADUREIRA, J., PACIÊNCIA, I., SOUSA, J. R. B., OLIVEIRA FERNANDES, E., SLEZAKOVA, K., PEREIRA, M. C., AGUIAR, L., TEIXEIRA, J. P., PINTO, M., DELGADO, L. & MOREIRA, A. 2016b. Exposure to emissions from cleaning products in primary schools: A test chamber study. *Occupational Safety and Hygiene IV.* CRC Press.

CAVALEIRO RUFO, J., MOREIRA, A. & DELGADO, L. 2017b. Reducing the burden of allergy and asthma in schoolchildren: Air cleaning solutions and microbial diversity-the dark side of the moon? *J Allergy Clin Immunol Pract,* 5**,** 1164-1165.

CAVALEIRO RUFO, J., PACIENCIA, I., SILVA, D., MARTINS, C., MADUREIRA, J., OLIVEIRA FERNANDES, E., PADRAO, P., MOREIRA, P., DELGADO, L. & MOREIRA, A. 2018. Swimming pool exposure is associated with autonomic changes and increased airway reactivity to a beta-2 agonist in school aged children: A cross-sectional survey. *PLoS One,* 13**,** e0193848.

CHIN, J. Y., GODWIN, C., PARKER, E., ROBINS, T., LEWIS, T., HARBIN, P. & BATTERMAN, S. 2014. Levels and sources of volatile organic compounds in homes of children with asthma. *Indoor Air,* 24**,** 403-15.

CHUNG, K. F. 2016. Asthma phenotyping: a necessity for improved therapeutic precision and new targeted therapies. *J Intern Med,* 279**,** 192-204.

COCKCROFT, D. W. 2010. Direct challenge tests: Airway hyperresponsiveness in asthma: its measurement and clinical significance. *Chest,* 138**,** 18s-24s.

COLSOUL, B., NILIUS, B. & VENNEKENS, R. 2009. On the putative role of transient receptor potential cation channels in asthma. *Clin Exp Allergy,* 39**,** 1456–1466.

CONTOLI, M. & PAPI, A. 2010. When asthma diagnosis becomes a challenge. *Eur Respir J,* 36**,** 231-3.

COURTEIX, D., OBERT, P., LECOQ, A.-M., GUENON, P. & KOCH, G. 1997. Effect of intensive swimming training on lung volumes, airway resistances and on the maximal expiratory flow-volume relationship in prepubertal girls. *European Journal of Applied Physiology and Occupational Physiology,* 76**,** 264-269.

COUTO, M., BARBOSA, C., SILVA, D., RUDNITSKAYA, A., DELGADO, L., MOREIRA, A. & ROCHA, S. M. 2017. Oxidative stress in asthmatic and non-asthmatic adolescent swimmers-A breathomics approach. *Pediatr Allergy Immunol,* 28**,** 452-457.

COUTO, M., SILVA, D., SANTOS, P., QUEIROS, S., DELGADO, L. & MOREIRA, A. 2015. Exploratory study comparing dysautonomia between asthmatic and non-asthmatic elite swimmers. *Rev Port Pneumol (2006),* 21**,** 22-9.

COVAR, R. A., SPAHN, J. D., MARTIN, R. J., SILKOFF, P. E., SUNDSTROM, D. A., MURPHY, J. & SZEFLER, S. J. 2004. Safety and application of induced sputum analysis in childhood asthma. *J Allergy Clin Immunol,* 114**,** 575-82.

DALLINGA, J. W., ROBROEKS, C. M., VAN BERKEL, J. J., MOONEN, E. J., GODSCHALK, R. W., JOBSIS, Q., DOMPELING, E., WOUTERS, E. F. & VAN SCHOOTEN, F. J. 2010. Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children. *Clin Exp Allergy,* 40**,** 68-76.

DANNEMILLER, K. C., MENDELL, M. J., MACHER, J. M., KUMAGAI, K., BRADMAN, A., HOLLAND, N., HARLEY, K., ESKENAZI, B. & PECCIA, J. 2014.

Next-generation DNA sequencing reveals that low fungal diversity in house dust is associated with childhood asthma development. *Indoor Air,* 24**,** 236-47.

DAS, S., PAL, S. & MITRA, M. 2016. Significance of Exhaled Breath Test in Clinical Diagnosis: A Special Focus on the Detection of Diabetes Mellitus. *Journal of Medical and Biological Engineering,* 36**,** 605-624.

DE LACY COSTELLO, B., AMANN, A., AL-KATEB, H., FLYNN, C., FILIPIAK, W., KHALID, T., OSBORNE, D. & RATCLIFFE, N. M. 2014. A review of the volatiles from the healthy human body. *J Breath Res,* 8**,** 014001.

DE LAURENTIIS, G., PARIS, D., MELCK, D., MANISCALCO, M., MARSICO, S., CORSO, G., MOTTA, A. & SOFIA, M. 2008. Metabonomic analysis of exhaled breath condensate in adults by nuclear magnetic resonance spectroscopy. *European Respiratory Journal,* 32**,** 1175-1183.

DELFINO, R. J., GONG, H., LINN, W. S., HU, Y. & PELLIZZARI, E. D. 2003. Respiratory symptoms and peak expiratory flow in children with asthma in relation to volatile organic compounds in exhaled breath and ambient air. *J Expo Anal Environ Epidemiol,* 13**,** 348-63.

DENT, J., EL-SERAG, H. B., WALLANDER, M. A. & JOHANSSON, S. 2005. Epidemiology of gastro-oesophageal reflux disease: a systematic review. *Gut,* 54**,** 710-717.

DINNES, J., DEEKS, J., KIRBY, J. & RODERICK, P. 2005. A methodological review of how heterogeneity has been examined in systematic reviews of diagnostic test accuracy. *Health Technol Assess,* 9**,** 1-113.

DOHERTY, M. & DIMITRIOU, L. 1997. Comparison of lung volume in Greek swimmers, land based athletes, and sedentary controls using allometric scaling. *British Journal of Sports Medicine,* 31**,** 337-341.

DOSA, D. M. 2007. A day in the life of Oscar the cat. *N Engl J Med,* 357**,** 328-9.

DRAGONIERI, S., SCHOT, R., MERTENS, B. J., LE CESSIE, S., GAUW, S. A., SPANEVELLO, A., RESTA, O., WILLARD, N. P., VINK, T. J., RABE, K. F., BEL, E. H. & STERK, P. J. 2007. An electronic nose in the discrimination of patients with asthma and controls. *J Allergy Clin Immunol,* 120**,** 856-62.

DWEIK, R. A., BOGGS, P. B., ERZURUM, S. C., IRVIN, C. G., LEIGH, M. W., LUNDBERG, J. O., OLIN, A. C., PLUMMER, A. L. & TAYLOR, D. R. 2011. An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications. *Am J Respir Crit Care Med,* 184**,** 602-15.

EGE, M. J., MAYER, M., NORMAND, A.-C., GENUNEIT, J., COOKSON, W. O. C. M., BRAUN-FAHRLÄNDER, C., HEEDERIK, D., PIARROUX, R. & VON MUTIUS, E. 2011. Exposure to Environmental Microorganisms and Childhood Asthma. *New England Journal of Medicine,* 364**,** 701-709.

EUROPEAN STANDARDS 2000. CSN EN 13098: Workplace atmosphere - Guidelines for measurement of airborne micro-organisms and endotoxin.

EUROSTAT 2014. Youth population on 1 January by sex, age and country of birth.

FENS, N., ROLDAAN, A. C., VAN DER SCHEE, M. P., BOKSEM, R. J., ZWINDERMAN, A. H., BEL, E. H. & STERK, P. J. 2011. External validation of exhaled breath profiling using an electronic nose in the discrimination of asthma with fixed airways obstruction and chronic obstructive pulmonary disease. *Clin Exp Allergy,* 41**,** 1371-8.

FENS, N., ZWINDERMAN, A. H., VAN DER SCHEE, M. P., DE NIJS, S. B., DIJKERS, E., ROLDAAN, A. C., CHEUNG, D., BEL, E. H. & STERK, P. J. 2009. Exhaled Breath Profiling Enables Discrimination of Chronic Obstructive Pulmonary Disease and Asthma. *Am. J. Respir. Crit. Care Med.,* 180**,** 1076-1082.

FILIPIAK, W., MOCHALSKI, P., FILIPIAK, A., AGER, C., CUMERAS, R., DAVIS, C. E., AGAPIOU, A., UNTERKOFLER, K. & TROPPMAIR, J. 2016. A Compendium of Volatile Organic Compounds (VOCs) Released By Human Cell Lines. *Current Medicinal Chemistry,* 23**,** 2112-2131.

FONT-RIBERA, L., KOGEVINAS, M., ZOCK, J. P., NIEUWENHUIJSEN, M. J., HEEDERIK, D. & VILLANUEVA, C. M. 2009. Swimming pool attendance and risk of asthma and allergic symptoms in children. *Eur Respir J,* 34**,** 1304-10.

FONT-RIBERA, L., VILLANUEVA, C. M., GRACIA-LAVEDAN, E., BORRAS-SANTOS, A., KOGEVINAS, M. & ZOCK, J. P. 2014. Indoor swimming pool attendance and respiratory and dermal health in schoolchildren--HITEA Catalonia. *Respir Med,* 108**,** 1056-9.

FONT-RIBERA, L., VILLANUEVA, C. M., NIEUWENHUIJSEN, M. J., ZOCK, J. P., KOGEVINAS, M. & HENDERSON, J. 2011. Swimming pool attendance, asthma, allergies, and lung function in the Avon Longitudinal Study of Parents and Children cohort. *Am J Respir Crit Care Med,* 183**,** 582-8.

FUJIMURA, K. E. & LYNCH, S. V. 2015. Microbiota in Allergy and Asthma and the Emerging Relationship with the Gut Microbiome. *Cell host & microbe,* 17**,** 592-602.

GAHLEITNER, F., GUALLAR-HOYAS, C., BEARDSMORE, C. S., PANDYA, H. C. & THOMAS, C. P. 2013. Metabolomics pilot study to identify volatile organic compound markers of childhood asthma in exhaled breath. *Bioanalysis,* 5**,** 2239-47.

GAN, H. L., MAN, Y. B. C., TAN, C. P., NORAINI, I. & NAZIMAH, S. A. H. 2005. Characterisation of vegetable oils by surface acoustic wave sensing electronic nose. *Food Chemistry,* 89**,** 507-518.

GEMICIOGLU, B., MUSELLIM, B., DOGAN, I. & GUVEN, K. 2014. Fractional exhaled nitric oxide (FeNo) in different asthma phenotypes. *Allergy & Rhinology,* 5**,** e157-e161.

GIACCO, S. R. D., FIRINU, D., BJERMER, L. & CARLSEN, K.-H. 2015. Exercise and asthma: an overview. *European Clinical Respiratory Journal,* 2**,** 10.3402/ecrj.v2.27984.

GLOBAL ASTHMA NETWORK. 2014. *The Global Asthma Report* [Online]. Available: http://www.globalasthmareport.org/burden/burden.php [Accessed 27 November 2017].

GLOBAL INITIATIVE FOR ASTHMA 2017. Global stategy for asthma management and prevention. 2017 ed.

GOODMAN, M. & HAYS, S. 2008. Asthma and swimming: a meta-analysis. *J Asthma,* 45**,** 639-47.

GREEN, G. C., CHAN, A. D. C., DAN, H. & LIN, M. 2011. Using a metal oxide sensor (MOS)-based electronic nose for discrimination of bacteria based on individual colonies in suspension. *Sensors and Actuators B: Chemical,* 152**,** 21-28.

HAAHTELA, T. 2014. What is needed for allergic children? *Pediatr Allergy Immunol,* 25**,** 21-4.

HIGGINS, J. P. T., THOMPSON, S. G., DEEKS, J. J. & ALTMAN, D. G. 2003. Measuring inconsistency in meta-analyses. *BMJ : British Medical Journal,* 327**,** 557-560.

HÜTTMANN, E.-M., GREULICH, T., HATTESOHL, A., SCHMID, S., NOESKE, S., HERR, C., JOHN, G., JÖRRES, R. A., MÜLLER, B., VOGELMEIER, C. & KOCZULLA, A. R. 2011. Comparison of Two Devices and Two Breathing Patterns for Exhaled Breath Condensate Sampling. *PLoS ONE,* 6**,** e27467.

IBRAHIM, B., BASANTA, M., CADDEN, P., SINGH, D., DOUCE, D., WOODCOCK, A. & FOWLER, S. J. 2011. Non-invasive phenotyping using exhaled volatile organic compounds in asthma. *Thorax,* 66**,** 804-9.

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION 2013. 4833-1: Microbiology of the food chain -- Horizontal method for the enumeration of microorganisms -- Part 1: Colony count at 30 degrees C by the pour plate technique.

IZQUIERDO-GARCÍA, J. L., PECES-BARBA, G., HEILI, S., DIAZ, R., WANT, E. & RUIZ-CABELLO, J. 2011. Is NMR-based metabolomic analysis of exhaled breath condensate accurate? *European Respiratory Journal,* 37**,** 468-470.

JACOBS, J. H., FUERTES, E., KROP, E. J., SPITHOVEN, J., TROMP, P. & HEEDERIK, D. J. 2012. Swimming pool attendance and respiratory symptoms and allergies among Dutch children. *Occup Environ Med,* 69**,** 823-30.

JACOBS, J. H., SPAAN, S., VAN ROOY, G. B., MELIEFSTE, C., ZAAT, V. A., ROOYACKERS, J. M. & HEEDERIK, D. 2007. Exposure to trichloramine and respiratory symptoms in indoor swimming pool workers. *Eur Respir J,* 29**,** 690-8.

JIANG, L., DIAZ, P. T., BEST, T. M., STIMPFL, J. N., HE, F. & ZUO, L. 2014. Molecular characterization of redox mechanisms in allergic asthma. *Ann Allergy Asthma Immunol,* 113**,** 137-42.

KARVONEN, A. M., HYVARINEN, A., GEHRING, U., KORPPI, M., DOEKES, G., RIEDLER, J., BRAUN-FAHRLANDER, C., BITTER, S., SCHMID, S., KESKI-NISULA, L., ROPONEN, M., KAULEK, V., DALPHIN, J. C., PFEFFERLE, P. I., RENZ, H., BUCHELE, G., VON MUTIUS, E. & PEKKANEN, J. 2012. Exposure to microbial agents in house dust and wheezing, atopic dermatitis and atopic sensitization in early childhood: a birth cohort study in rural areas. *Clin Exp Allergy,* 42**,** 1246-56.

KARVONEN, A. M., HYVARINEN, A., RINTALA, H., KORPPI, M., TAUBEL, M., DOEKES, G., GEHRING, U., RENZ, H., PFEFFERLE, P. I., GENUNEIT, J., KESKI-NISULA, L., REMES, S., LAMPI, J., VON MUTIUS, E. & PEKKANEN, J. 2014. Quantity and diversity of environmental microbial exposure and development of asthma: a birth cohort study. *Allergy,* 69**,** 1092-101.

KHARITONOV, S. A. & BARNES, P. J. 2001. Exhaled markers of pulmonary disease. *Am J Respir Crit Care Med,* 163**,** 1693-722.

KIM, M. H., SONG, W. J., KIM, T. W., JIN, H. J., SIN, Y. S., YE, Y. M., KIM, S. H., PARK, H. W., LEE, B. J., PARK, H. S., YOON, H. J., CHOI, D. C., MIN, K. U. & CHO, S. H. 2014. Diagnostic properties of the methacholine and mannitol bronchial challenge tests: a comparison study. *Respirology,* 19**,** 852-6.

KONRADSEN, J. R., CAFFREY OSVALD, E. & HEDLIN, G. 2015. Update on the current methods for the diagnosis and treatment of severe childhood asthma. *Expert Review of Respiratory Medicine,* 9**,** 769-777.

KRILAVICIUTE, A., HEISS, J. A., LEJA, M., KUPCINSKAS, J., HAICK, H. & BRENNER, H. 2015. Detection of cancer through exhaled breath: a systematic review. *Oncotarget,* 6**,** 38643-38657.

LÄRSTAD, M. A. E., TORÉN, K., BAKE, B. & OLIN, A. C. 2007. Determination of ethane, pentane and isoprene in exhaled air – effects of breath-holding, flow rate and purified air. *Acta Physiol,* 189**,** 87-98.

LAZOVIC-POPOVIC, B., ZLATKOVIC-SVENDA, M., DURMIC, T., DJELIC, M., DJORDJEVIC SARANOVIC, S. & ZUGIC, V. 2016. Superior lung capacity in swimmers: Some questions, more answers! *Revista Portuguesa de Pneumologia (English Edition),* 22**,** 151-156.

LEMANSKE, R. F. & BUSSE, W. W. 2010. Asthma: Clinical Expression and Molecular Mechanisms. *The Journal of allergy and clinical immunology,* 125**,** S95-102.

LEMANSKE, R. F., JR. & BUSSE, W. W. 2003. 6. Asthma. *J Allergy Clin Immunol,* 111**,** S502-19.

LIM, S. Y., JO, Y. J. & CHUN, E. M. 2014. The correlation between the bronchial hyperresponsiveness to methacholine and asthma like symptoms by GINA questionnaires for the diagnosis of asthma. *BMC Pulm. Med.,* 14**,** 161.

LIU, J. & THOMAS, P. S. 2007. Relationship between exhaled breath condensate volume and measurements of lung volumes. *Respiration,* 74**,** 142-5.

LODRUP CARLSEN, K. C., HEDLIN, G., BUSH, A., WENNERGREN, G., DE BENEDICTIS, F. M., DE JONGSTE, J. C., BARALDI, E., PEDROLETTI, C., BARBATO, A., MALMSTROM, K., POHUNEK, P., PEDERSEN, S., PIACENTINI, G. L., MIDDELVELD, R. J. & CARLSEN, K. H. 2011. Assessment of problematic severe asthma in children. *Eur Respir J,* 37**,** 432-40.

LOMAX, M. 2016. Airway dysfunction in elite swimmers: prevalence, impact, and challenges. *Open Access Journal of Sports Medicine,* 7**,** 55-63.

LORWONGTRAGOOL, P., SEESAARD, T., TONGTA, C. & KERDCHAROEN, T. Portable e-nose based on polymer/CNT sensor array for protein-based detection. 2012 7th IEEE International Conference on Nano/Micro Engineered and Molecular Systems (NEMS), 5-8 March 2012 2012. 1-6.

LOUDON, I. 1994. Medicine and the five senses. *Medical History,* 38**,** 226-227.

MACNEE, W. & DONALDSON, K. 2003. Mechanism of lung injury caused by PM10 and ultrafine particles with special reference to COPD. *Eur Respir J Suppl,* 40**,** 47s-51s.

MADUREIRA, J., PACIÊNCIA, I., RUFO, J. C., PEREIRA, C., TEIXEIRA, J. P. & DE OLIVEIRA FERNANDES, E. 2015. Assessment and determinants of airborne bacterial and fungal concentrations in different indoor environments: Homes, child day-care centres, primary schools and elderly care centres. *Atmospheric Environment,* 109**,** 139-146.

MADUREIRA, J., PEREIRA, C., PACIÊNCIA, I., TEIXEIRA, J. P. & DE OLIVEIRA FERNANDES, E. 2014. Identification and Levels of Airborne Fungi in Portuguese Primary Schools. *J. Toxicol. Env. Health Part A,* 77**,** 816-826.

MANOHARAN, A., ANDERSON, W. J., LIPWORTH, J. & LIPWORTH, B. J. 2015. Assessment of spirometry and impulse oscillometry in relation to asthma control. *Lung,* 193**,** 47-51.

MARTINS, C., SILVA, D., SEVERO, M., RUFO, J., PACIENCIA, I., MADUREIRA, J., PADRAO, P., MOREIRA, P., DELGADO, L., OLIVEIRA FERNANDES, E., BARROS, H., MALMBERG, P. & MOREIRA, A. 2017. Spirometry adjusted exhaled

nitric oxide increases accuracy for assessment of asthma control in children. *Pediatr Allergy Immunol*.

MENDELL, M. J., MIRER, A. G., CHEUNG, K., TONG, M. & DOUWES, J. 2011. Respiratory and allergic health effects of dampness, mold, and dampness-related agents: a review of the epidemiologic evidence. *Environ Health Perspect,* 119**,** 748-56.

MENDY, A., GASANA, J., VIEIRA, E. R., FORNO, E., PATEL, J., KADAM, P. & RAMIREZ, G. 2011. Endotoxin Exposure and Childhood Wheeze and Asthma: A Meta-Analysis of Observational Studies. *Journal of Asthma,* 48**,** 685-693.

MIEKISCH, W., SCHUBERT, J. K. & NOELDGE-SCHOMBURG, G. F. 2004. Diagnostic potential of breath analysis--focus on volatile organic compounds. *Clin Chim Acta,* 347**,** 25-39.

MILLER, M. R., HANKINSON, J., BRUSASCO, V., BURGOS, F., CASABURI, R., COATES, A., CRAPO, R., ENRIGHT, P., VAN DER GRINTEN, C. P., GUSTAFSSON, P., JENSEN, R., JOHNSON, D. C., MACINTYRE, N., MCKAY, R., NAVAJAS, D., PEDERSEN, O. F., PELLEGRINO, R., VIEGI, G. & WANGER, J. 2005. Standardisation of spirometry. *Eur Respir J,* 26**,** 319-38.

MLAKAR, A. & SPITELLER, G. 1994. Reinvestigation of lipid peroxidation of linolenic acid. *Biochim Biophys Acta,* 1214**,** 209-20.

MOCHALSKI, P., WZOREK, B., SLIWKA, I. & AMANN, A. 2009. Suitability of different polymer bags for storage of volatile sulphur compounds relevant to breath analysis. *J Chromatogr B Analyt Technol Biomed Life Sci,* 877**,** 189-96.

MOHER, D., LIBERATI, A., TETZLAFF, J., ALTMAN, D. G. & THE, P. G. 2009. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. *PLoS Med,* 6**,** e1000097.

MONTUSCHI, P. 2007. Analysis of exhaled breath condensate in respiratory medicine: methodological aspects and potential clinical applications. *Ther Adv Respir Dis,* 1**,** 5-23.

MONTUSCHI, P., SANTONICO, M., MONDINO, C., PENNAZZA, G., MANTINI, G., MARTINELLI, E., CAPUANO, R., CIABATTONI, G., PAOLESSE, R., DI NATALE, C., BARNES, P. J. & D'AMICO, A. 2010. Diagnostic performance of an

electronic nose, fractional exhaled nitric oxide, and lung function testing in asthma. *Chest,* 137**,** 790-6.

MORAWSKA, L., AFSHARI, A., BAE, G. N., BUONANNO, G., CHAO, C. Y. H., HÄNNINEN, O., HOFMANN, W., ISAXON, C., JAYARATNE, E. R., PASANEN, P., SALTHAMMER, T., WARING, M. & WIERZBICKA, A. 2013. Indoor aerosols: from personal exposure to risk assessment. *Indoor Air,* 23**,** 462-487.

MUPPIDI, S., ADAMS-HUET, B., TAJZOY, E., SCRIBNER, M., BLAZEK, P., SPAETH, E. B., FROHMAN, E., DAVIS, S. & VERNINO, S. 2013. Dynamic pupillometry as an autonomic testing tool. *Clin Auton Res,* 23**,** 297-303.

MURRAY, C. S., WOODCOCK, A. & CUSTOVIC, A. 2001. The role of indoor allergen exposure in the development of sensitization and asthma. *Curr Opin Allergy Clin Immunol,* 1**,** 407-12.

NATIONAL INSTITUTE FOR OCCUPATIONAL SAFETY AND HEALTH 1998. *NIOSH Manual of Analytical Methods*.

NEERINCX, A. H., VIJVERBERG, S. J. H., BOS, L. D. J., BRINKMAN, P., VAN DER SCHEE, M. P., DE VRIES, R., STERK, P. J. & MAITLAND-VAN DER ZEE, A. H. 2017. Breathomics from exhaled volatile organic compounds in pediatric asthma. *Pediatr Pulmonol,* 52**,** 1616-1627.

NICOLAOU, N., YIALLOUROS, P., PIPIS, S., IOANNOU, P., SIMPSON, A. & CUSTOVIC, A. 2006. Domestic allergen and endotoxin exposure and allergic sensitization in Cyprus. *Pediatr Allergy Immunol,* 17**,** 17-21.

NURMATOV, U. B., TAGIYEVA, N., SEMPLE, S., DEVEREUX, G. & SHEIKH, A. 2015. Volatile organic compounds and risk of asthma and allergy: a systematic review. *Eur Respir Rev,* 24**,** 92-101.

OBER, C. & HOFFJAN, S. 2006. Asthma genetics 2006: the long and winding road to gene discovery. *Genes Immun,* 7**,** 95-100.

OLOPADE, C. O., ZAKKAR, M., SWEDLER, W. I. & RUBINSTEIN, I. 1997. Exhaled pentane levels in acute asthma. *Chest,* 111**,** 862-5.

PACIENCIA, I., MADUREIRA, J., RUFO, J., MOREIRA, A. & FERNANDES EDE, O. 2016. A systematic review of evidence and implications of spatial and seasonal

variations of volatile organic compounds (VOC) in indoor human environments. *J Toxicol Environ Health B Crit Rev,* 19**,** 47-64.

PAREDI, P., KHARITONOV, S. A. & BARNES, P. J. 2000. Elevation of exhaled ethane concentration in asthma. *Am J Respir Crit Care Med,* 162**,** 1450-4.

PRASAD, A., LANGFORD, B., STRADLING, J. R. & HO, L.-P. 2006. Exhaled nitric oxide as a screening tool for asthma in school children. *Respiratory Medicine,* 100**,** 167-173.

RAMALHO, R., PIRRACO, A., SOARES, R., PALMARES, C., DELGADO, L. & MOREIRA, A. 2014. Neurogenic inflammation in the airways of elite swimmers. *J Sports Med Phys Fitness,* 54**,** 252-3.

ROBROEKS, C. M., VAN BERKEL, J. J., JÖBSIS, Q., VAN SCHOOTEN, F.-J., DALLINGA, J. W., WOUTERS, E. F. & DOMPELING, E. 2013. Exhaled volatile organic compounds predict exacerbations of childhood asthma in a 1-year prospective study. *Eur Respir J,* 42**,** 98-106.

ROMAGNOLI, M., VACHIER, I., TARODO DE LA FUENTE, P., MEZIANE, H., CHAVIS, C., BOUSQUET, J., GODARD, P. & CHANEZ, P. 2002. Eosinophilic inflammation in sputum of poorly controlled asthmatics. *Eur Respir J,* 20**,** 1370-7.

RUFO, J. C., MADUREIRA, J., FERNANDES, E. O. & MOREIRA, A. 2016. Volatile organic compounds in asthma diagnosis: a systematic review and meta-analysis. *Allergy,* 71**,** 175-88.

RUOKOLAINEN, L., VON HERTZEN, L., FYHRQUIST, N., LAATIKAINEN, T., LEHTOMAKI, J., AUVINEN, P., KARVONEN, A. M., HYVARINEN, A., TILLMANN, V., NIEMELA, O., KNIP, M., HAAHTELA, T., PEKKANEN, J. & HANSKI, I. 2015. Green areas around homes reduce atopic sensitization in children. *Allergy,* 70**,** 195-202.

SARMENTO, A. D. O., SANTOS, A. D. C., TROMBETTA, I. C., DANTAS, M. M., OLIVEIRA MARQUES, A. C., DO NASCIMENTO, L. S., BARBOSA, B. T., DOS SANTOS, M. R., ANDRADE, M. D. A., JAGUARIBE-LIMA, A. M. & BRASILEIRO-SANTOS, M. D. S. 2017. Regular physical exercise improves cardiac autonomic and muscle vasodilatory responses to isometric exercise in healthy elderly. *Clinical Interventions in Aging,* 12**,** 1021-1028.

SCHIVO, M., SEICHTER, F., AKSENOV, A. A., PASAMONTES, A., PEIRANO, D. J., MIZAIKOFF, B., KENYON, N. J. & DAVIS, C. E. 2013. A mobile instrumentation platform to distinguish airway disorders. *J Breath Res,* 7**,** 017113.

SCHNEIDER, A., GINDNER, L., TILEMANN, L., SCHERMER, T., DINANT, G. J., MEYER, F. J. & SZECSENYI, J. 2009. Diagnostic accuracy of spirometry in primary care. *BMC Pulm Med,* 9**,** 31.

SCHWARTZ, J., LITONJUA, A., SUH, H., VERRIER, M., ZANOBETTI, A., SYRING, M., NEARING, B., VERRIER, R., STONE, P., MACCALLUM, G., SPEIZER, F. & GOLD, D. 2005. Traffic related pollution and heart rate variability in a panel of elderly subjects. *Thorax,* 60**,** 455-461.

SHARPE, R. A., BEARMAN, N., THORNTON, C. R., HUSK, K. & OSBORNE, N. J. 2015. Indoor fungal diversity and asthma: a meta-analysis and systematic review of risk factors. *J Allergy Clin Immunol,* 135**,** 110-22.

SILVA, D. & MOREIRA, A. 2015. The role of sports and exercise in allergic disease: drawbacks and benefits. *Expert Rev Clin Immunol,* 11**,** 993-1003.

SIMEL, D. L., SAMSA, G. P. & MATCHAR, D. B. 1991. Likelihood ratios with confidence: sample size estimation for diagnostic test studies. *J Clin Epidemiol,* 44**,** 763-70.

SMITH, A. D., COWAN, J. O., FILSELL, S., MCLACHLAN, C., MONTI-SHEEHAN, G., JACKSON, P. & TAYLOR, D. R. 2004. Diagnosing asthma: comparisons between exhaled nitric oxide measurements and conventional tests. *Am J Respir Crit Care Med,* 169**,** 473-8.

SMOLINSKA, A., KLAASSEN, E. M., DALLINGA, J. W., VAN DE KANT, K. D., JOBSIS, Q., MOONEN, E. J., VAN SCHAYCK, O. C., DOMPELING, E. & VAN SCHOOTEN, F. J. 2014. Profiling of volatile organic compounds in exhaled breath as a strategy to find early predictive signatures of asthma in children. *PLoS One,* 9**,** e95668.

SPAHN, J. D. 2012. Asthma biomarkers in sputum. *Immunol Allergy Clin North Am,* 32**,** 387-99.

STANG, J., COUTO, M., STENSRUD, T., MOWINCKEL, P., MOREIRA, A. & CARLSEN, K. H. 2016. Assessment of Parasympathetic Activity in Athletes: Comparing Two Different Methods. *Med Sci Sports Exerc,* 48**,** 316-22.

STERN, D. A., MORGAN, W. J., HALONEN, M., WRIGHT, A. L. & MARTINEZ, F. D. 2008. Wheezing and bronchial hyper-responsiveness in early childhood as predictors of newly diagnosed asthma in early adulthood: a longitudinal birth-cohort study. *Lancet,* 372**,** 1058-64.

THORNE, P. S., KULHANKOVA, K., YIN, M., COHN, R., ARBES, S. J., JR. & ZELDIN, D. C. 2005. Endotoxin exposure is a risk factor for asthma: the national survey of endotoxin in United States housing. *Am J Respir Crit Care Med,* 172**,** 1371-7.

TIMMS, C., THOMAS, P. S. & YATES, D. H. 2012. Detection of gastro-oesophageal reflux disease (GORD) in patients with obstructive lung disease using exhaled breath profiling. *J Breath Res,* 6**,** 1752-7155.

TSE, S. M., GOLD, D. R., SORDILLO, J. E., HOFFMAN, E. B., GILLMAN, M. W., RIFAS-SHIMAN, S. L., FUHLBRIGGE, A. L., TANTISIRA, K. G., WEISS, S. T. & LITONJUA, A. A. 2013. Diagnostic accuracy of the bronchodilator response in children. *J Allergy Clin Immunol,* 132**,** 554-559.e5.

UYAN, Z. S., CARRARO, S., PIACENTINI, G. & BARALDI, E. 2009. Swimming pool, respiratory health, and childhood asthma: should we change our beliefs? *Pediatr Pulmonol,* 44**,** 31-7.

VAN BERKEL, J. J., DALLINGA, J. W., MOLLER, G. M., GODSCHALK, R. W., MOONEN, E., WOUTERS, E. F. & VAN SCHOOTEN, F. J. 2008. Development of accurate classification method based on the analysis of volatile organic compounds from human exhaled air. *J Chromatogr B Analyt Technol Biomed Life Sci,* 861**,** 101-7.

VAN DE KANT, K. D., VAN DER SANDE, L. J., JÖBSIS, Q., VAN SCHAYCK, O. C. & DOMPELING, E. 2012. Clinical use of exhaled volatile organic compounds in pulmonary diseases: a systematic review. *Respiratory Research,* 13**,** 117.

VAN DEN AKKER-VAN MARLE, M. E., BRUIL, J. & DETMAR, S. B. 2005. Evaluation of cost of disease: assessing the burden to society of asthma in children in the European Union. *Allergy,* 60**,** 140-9.

VAN DER SCHEE, M. P., HASHIMOTO, S., SCHUURMAN, A. C., VAN DRIEL, J. S., ADRIAENS, N., VAN AMELSFOORT, R. M., SNOEREN, T., REGENBOOG, M., SPRIKKELMAN, A. B., HAARMAN, E. G., VAN AALDEREN, W. M. & STERK, P. J. 2015a. Altered exhaled biomarker profiles in children during and after rhinovirus-induced wheeze. *Eur Respir J,* 45**,** 440-8.

VAN DER SCHEE, M. P., PAFF, T., BRINKMAN, P., VAN AALDEREN, W. M. C., HAARMAN, E. G. & STERK, P. J. 2015b. Breathomics in lung disease. *Chest,* 147**,** 224-231.

VAN DER SCHEE, M. P., PALMAY, R., COWAN, J. O. & TAYLOR, D. R. 2013. Predicting steroid responsiveness in patients with asthma using exhaled breath profiling. *Clin Exp Allergy,* 43**,** 1217-25.

VOISIN, C., SARDELLA, A., MARCUCCI, F. & BERNARD, A. 2010. Infant swimming in chlorinated pools and the risks of bronchiolitis, asthma and allergy. *Eur Respir J,* 36**,** 41-7.

WANG, C., SALAM, M. T., ISLAM, T., WENTEN, M., GAUDERMAN, W. J. & GILLILAND, F. D. 2008. Effects of in utero and childhood tobacco smoke exposure and beta2-adrenergic receptor genotype on childhood asthma and wheezing. *Pediatrics,* 122**,** e107-14.

WENZEL, S. E. & BUSSE, W. W. 2007. Severe asthma: Lessons from the Severe Asthma Research Program. *Journal of Allergy and Clinical Immunology,* 119**,** 14-21.

WHITING, P., RUTJES, A., REITSMA, J., BOSSUYT, P. & KLEIJNEN, J. 2003. The development of QUADAS: a tool for the quality assessment of studies of diagnostic accuracy included in systematic reviews. *BMC Medical Research Methodology,* 3**,** 25.

WILLIAMS, A. E. 2012. *Immunology: Mucosal and Body Surface Defences,* UK, Wiley-Blackwell.

WILLIAMS, H. C., BURNEY, P. G., PEMBROKE, A. C. & HAY, R. J. 1994. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. III. Independent hospital validation. *Br J Dermatol,* 131**,** 406-16.

WILSON, A. D. 2015. Advances in electronic-nose technologies for the detection of volatile biomarker metabolites in the human breath. *Metabolites,* 5**,** 140-63.

WILSON, A. D. & BAIETTO, M. 2009. Applications and advances in electronic-nose technologies. *Sensors (Basel),* 9**,** 5099-148.

WILSON, A. D. & BAIETTO, M. 2011. Advances in electronic-nose technologies developed for biomedical applications. *Sensors (Basel),* 11**,** 1105-76.

YANG, I. V., LOZUPONE, C. A. & SCHWARTZ, D. A. 2017. The environment, epigenome, and asthma. *J Allergy Clin Immunol,* 140**,** 14-23.

YOO, Y. J., YANG, H. K. & HWANG, J. M. 2017. Efficacy of digital pupillometry for diagnosis of Horner syndrome. *PLoS One,* 12**,** e0178361.

ZAMORA, J., ABRAIRA, V., MURIEL, A., KHAN, K. & COOMARASAMY, A. 2006. Meta-DiSc: a software for meta-analysis of test accuracy data. *BMC Medical Research Methodology,* 6**,** 31.

ZRODNIKOV, Y. & DAVIS, C. E. 2012. The Highs and Lows of FAIMS: Predictions and Future Trends for High Field Asymmetric Waveform Ion Mobility Spectrometry. *Journal of nanomedicine & nanotechnology,* 3**,** 109e.

Publications

Study I

J. Cavaleiro Rufo, J. Madureira, I. Paciência, L. Aguiar, C. Pereira, D. Silva, P. Padrão, P. Moreira, L. Delgado, I. Annesi-Maesano, E. Oliveira Fernandes, J. P. Teixeira, A. Moreira. Indoor fungal diversity in primary schools may differently influence allergic sensitization and asthma in children.

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ORIGINAL ARTICLE Lower Airways

Indoor fungal diversity in primary schools may differently influence allergic sensitization and asthma in children

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Keywords
endotoxins; exposure; fungi; indoor air; microbiologic diversity; microbiome; schools; sensitization

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Abstract

Background: Childhood exposure to microbiologic agents may influence the development of allergic and respiratory diseases. Apart from home, children spend most of their time at school, which represents an environment of significant exposure to indoor air microbes. Therefore, we aimed to assess how the prevalence of allergic sensitization and asthma in schoolchildren is affected by microbiologic exposure within classrooms.

Methods: Spirometry with bronchodilation, exhaled nitric oxide measurements and skin-prick tests data were retrieved from 858 children aged 8–10 years attending 71 classrooms in 20 primary schools. Air samples were collected in all classrooms using a single-stage microbiologic air impactor through agar plates. Gram-negative endotoxins were collected using flow control pumps and analysed by limulus amebocyte lysate assay. Diversity scores were established as the number of different fungal species found in each classroom.

Results: Classrooms with increased diversity scores showed a significantly lower prevalence of children with atopic sensitization, but not asthma. The risk of sensitization increased with increasing endotoxin exposure in classrooms. Similarly, significantly higher concentrations of *Penicillium spp* were found in classrooms with a higher number of children with atopic sensitization.

Conclusions: Although no causal relationships could be established, exposure to higher fungal diversity was protective against allergic sensitization but this was not seen for asthma. In contrast, higher exposure to Gram-negative endotoxins and *Penicillium spp* in primary school's classrooms was associated with increasing odds of allergic sensitization in children.

Exposure to indoor air microbiologic agents during childhood has been shown to influence the development of allergic diseases (1). While there is evidence that excessive exposure to indoor air bacteria or fungi concentrations may induce the development of allergy and asthma (2–4), a large number of recent studies also suggest that exposure to certain microorganisms in the early years of life may have a protective effect in children, reducing the risk of allergy and asthma onset by promoting the maturation of Th1 lymphocytes, thus decreasing the incidence of Th2-oriented immunity (hygiene hypothesis) (5–9). There is published evidence supporting the general concept that a low diversity of the human microbiome during infancy is associated with the long-term development of lifestyle-dependent immune disease manifestations, such as atopic disease (10).

Apart from home, young children spend a large section of their daytime at school, mostly within their classrooms, which might be reflected as a long-term exposure to indoor air

microbes (5, 11). If the human microbiome does change according to environmental exposure, the classroom environment might contribute to microbiome changes and, consequently, either induce the development and/or exacerbation of allergy and asthma or, on the other hand, have a protective effect against their onset.

Therefore, the aim of this cross-sectional study was to observe how the prevalence of allergic sensitization and asthma in schoolchildren is influenced by the exposure to indoor air bacteria and fungi in classrooms. More specifically, this work aimed to (i) quantify the concentration of microbiologic parameters in the indoor air of primary school classrooms; and (ii) investigate how microbial concentrations and diversity may influence the risk of asthma and allergy in primary school children.

Methods

Study design and participants

A total of 20 public primary schools located in the city of Porto, Portugal, were invited to participate in this cross-sectional survey, corresponding to a total of 71 assessed classrooms. The sampling campaigns occurred during the heating season in two different periods: from January to April 2014, and from October 2014 to March 2015 (10 schools per period). The heating season was chosen as the air exchange rates in schools are usually lower during this period. During the sampling, indoor air bacteria, fungi and lipopolysaccharides (LPS) were collected. Concurrently, the clinical assessment of the participating children was performed in the school.

All schools where the study was carried out, along with the Ethical Committee of the University of Porto, approved the study protocol. Children attending the 71 participating classrooms were invited to take part in the study, corresponding to 1602 invited children (Fig. 1). After receiving the written consent from their legal guardians, 916 children were enrolled in the study (participation rate of 57%). Considering a confidence level of 95%, the estimated confidence interval for the sample size was 2.42, showing that the study has a good statistical power. A total of 58 children refused to perform the clinical tests having only the questionnaire data available; thus, spirometry, skin-prick testing and exhaled nitric oxide data were retrieved from 858 children (mean age of 9 ± 1 years).

Clinical assessment

In all participating children, height, weight, lung function (spirometry with bronchodilation) and exhaled level of nitric oxide (NO) were measured and skin-prick tests (SPTs) performed by a trained professional. The parents fulfilled a questionnaire based on the International Study of Asthma and Allergies in Childhood (ISAAC) where they reported the children's symptoms history.

Lung function and airway reversibility were assessed according to the official ATS/ERS guidelines (12). Lung function data were retrieved before and 15 min after 400 μg of inhaled salbutamol.

Airway inflammation was assessed measuring exhaled NO levels using the NObreath (Bedfont Scientific Ltd., Harrietsham, Kent, UK). The results were expressed as parts per billion (ppb) and stratified according to the official ATS guidelines for children (13).

Allergic sensitization was evaluated by SPT on their forearm using a QuickTest™ (Panatrex Inc., Placentia, California, USA) applicator containing *Der p*, mix of weeds (*Urtica dioica*, *Plantago lanceolate* and *Artemisia vulgaris*), mix of grasses (*Agrostis stolonifera*, *Anthoxathum odoratum*, *Dactylis glomerata*, *Lolium perenne*, *Arrhenatherium elatius*, *Festuca rubra*, *Poa pratensis*, *Holcus lanatus*, *Phleum pratense*, *Secale cereal*), cat dander, dog dander and *Alt a*, negative control (extracts dilutant) and a positive control (histamine at 10 mg/ml), all belonging to the same batches (Hall Allergy, Netherlands). Results were read 20 min afterwards and allergic sensitization was defined by a positive SPT to at least one of the allergens. If children were on antihistamines or topical corticosteroids on the skin within the previous 7 days, SPTs would be postponed.

The following operational asthma definitions were adopted: (i) clinical criteria – at least a 12% increase in FEV_1 after bronchodilation and over 200 ml and/or asthma diagnosed by a physician with reported symptoms (wheezing, dyspnoea or dry cough) occurring in the past 12 months; (ii) functional criteria – at least a 12% increase in FEV_1 after bronchodilation and over 200 ml; (iii) treated asthma criteria – asthma diagnosed by a physician and currently under inhaled corticosteroid treatment; and iv) ever asthma – asthma diagnosed by a physician.

Children were classified as having allergic rhinitis when their legal guardians answered positively to the question 'Did the participant suffer from recurrent sneezing, rhinorrhea or nasal congestion in the last 12 months, while not having a cold or flu?'. Atopic dermatitis was defined in accordance with the United Kingdom Working Party's Diagnostic Criteria for Atopic Dermatitis (14). The 'allergic disease' group was comprised by individuals with positive SPT suffering from allergic rhinitis and/or atopic dermatitis.

Characterization of the participants is presented in Table 1. Prevalence of asthma diagnosed by clinical criteria, functional criteria, treated asthma and ever asthma was 9.3%, 6.6%, 5.4% and 6.3%, respectively. Prevalence of allergic sensitization was 34.1%. The prevalence of allergic rhinitis and atopic dermatitis was 10.6% and 9.3%, respectively.

Microorganism sampling

Bacterial and fungal air samples were collected using a single-stage microbiologic air impactor (Merck Air Sampler MAS-10 0), according to NIOSH method 0800 (15) and EN 13098 (16). Tryptic soy agar (supplemented with 0.25% cycloheximide) and malt extract agar (supplemented with 1% of chloramphenicol) were used as culture media for bacteria and fungi, respectively. Air was drawn through the sampler at a 100 l/min rate and sequential duplicate air samples of 250 l were collected. Indoor air samples were obtained from the 71 participating classrooms. A total of two tryptic soy agar and two malt extract agar samples were collected per classroom. The mean CFU values of duplicate samples were used as the final

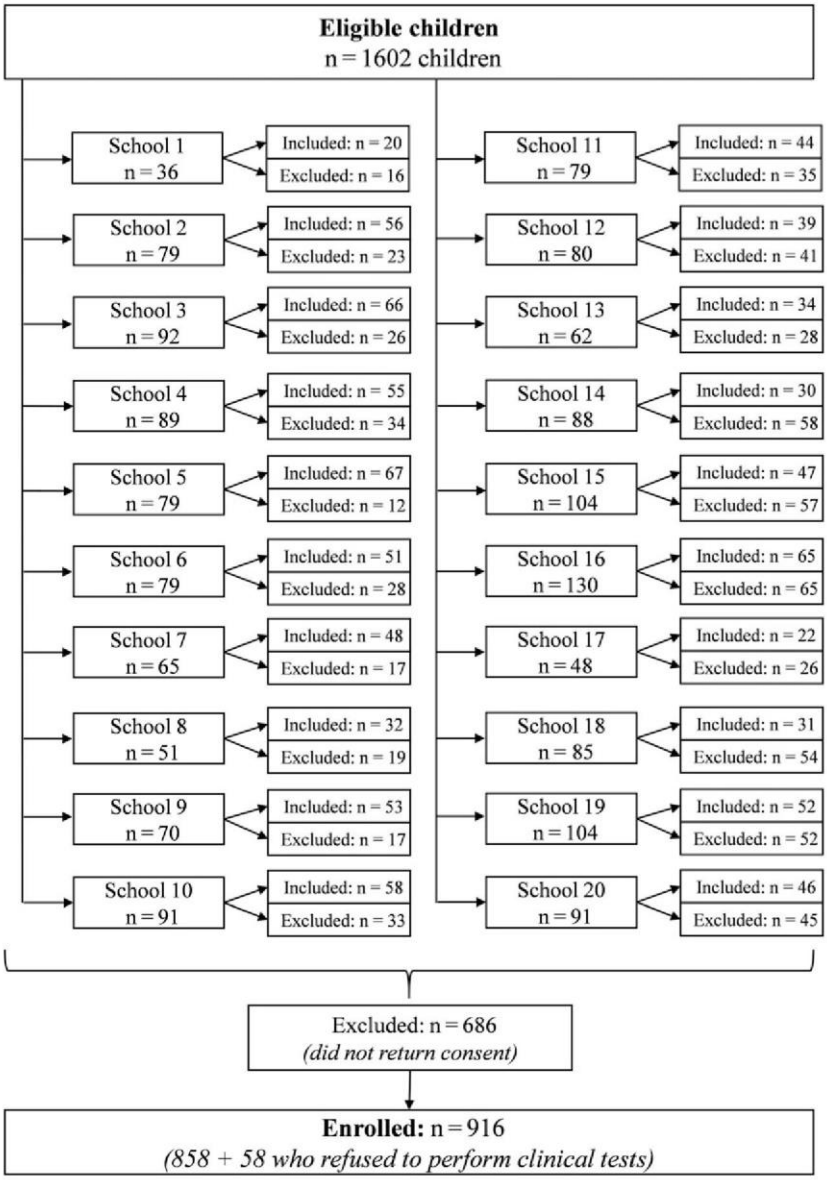

Figure 1 Flow of study participants.

result in accordance with the laboratory criteria. The volume, and consequently the duration, of sequential air sampling was the same in all schools and all classrooms. In each sampling day, four field blanks, two sterility blanks, one positive and one negative control per culture medium were used. This methodology has been validated in other works (17, 18).

Concurrently with bacteria and fungi assessment, indoor air LPS samples were collected during 4 h with GilAir-5 flow control pumps (Sensidyne, St. Petersburg, Florida, USA) set to 2 l/min and coupled to button aerosol stainless steel samplers (SKC Inc., Valley View Road Eighty Four, Pennsylvania, USA).

Laboratory analysis

Bacterial and fungal samples were incubated at 37 ± 1°C for 48 ± 3 h and at 25 ± 3°C for 72 ± 3 h, respectively (16–18). Quantification of bacteria and fungi levels was performed by naked eye count following an internal procedure based on the methodologies expressed in EN 13098 (16) and ISO 4833-1:2013 (19). The number of colonies recovered on the air sample plates was adjusted using a positive hole correction factor, and the results were expressed as number of colony-forming units per cubic metre of air (CFU/m3). The correction factor was based on Fellers law. The quantification limit was established as 10 CFU per plate.

Specific fungal identification was performed 7 days after incubation, either on the original sampling media-MEA plates or after subculturing procedures, whenever colony isolation and growth observation were needed. Identification of fungal colonies was based upon phenotypic characteristics and followed standard mycological procedures based on their micro and macro-morphological characteristics.

Table 1 Characteristics of the participants

	Total n = 858	Female n = 427	Male n = 431
Age (years, mean ± sd)	9 ± 1	9 ± 1	9 ± 1
Weight (kg)	31.2 (27.3–37.2)	31.8 (27.0–37.3)	30.8 (27.5–36.8)
Height (cm)	135 (130–140)	135 (130–139)	135 (130–140)
BMI (kg/m^2)	17.0 (15.5–19.5)	17.3 (15.6–19.8)	16.8 (15.5–19.2)
Clinical			
Allergic sensitization (n)	293	139	154
Asthma (n)	73	43	30
Allergic rhinitis (n)	91	37	54
Atopic dermatitis (n)	80	37	43
Allergic disease (n)*	101	40	61
Lung function			
FEV_1 (l)	1.75 (1.58–1.95)	1.71 (1.55–1.92)	1.77 (1.59–1.99)
FVC (l)	1.89 (1.69–2.14)	1.83 (1.66–2.08)	1.94 (1.72–2.18)
FEF_{25-75} (l/s)	2.28 (1.92–2.65)	2.28 (1.93–2.64)	2.29 (1.91–2.68)
FEV1/FVC (%)	92.7 (88.9–96.4)	93.2 (89.8–96.6)	92.0 (88.0–96.1)
FEV_1 reversibility (%)	3.5 (0.0–7.1)	3.7 (0.0–7.2)	3.5 (0.5–7.0)
FEV_1 reversibility (ml)	60 (0–120)	60 (0–120)	60 (10–130)
Exhaled NO (ppb)	11.0 (6.0–20.0)	9.5 (5.0–16.0)	12.0 (6.0–22.5)

BMI, body mass index; FEV_1, forced expiratory volume in the first second of FVC; FVC, forced vital capacity; FEF_{25-75}, forced expiratory flow middle portion of FVC.

*The "allergic disease" group was comprised by individuals with positive SPT suffering from allergic rhinitis and/or atopic dermatitis.

Data reported as median (25–75%) unless otherwise stated.

For endotoxin extraction, sample filters were eluted in 5 ml extraction solution (Pyrogen Free Water plus 0.05% Tween-20) and rocked vigorously for 1 h at room temperature on a horizontal shaker. After 10 min of centrifugation at 1000 ***g***, total supernatant per sample was collected and analysed. Endotoxin quantification was performed using the limulus amebocyte lysate (LAL) Kinetic-QCLTM (Lonza®, Pontevedra, Spain) following the manufacturer's guidelines. Endotoxin concentrations were expressed as EU/m^3. The limit of detection for the LAL Kinetic-QCLTM is 0.005 EU/ml, corresponding to 0.025 EU/m^3 under the adopted procedure.

Statistical analysis

The SPSS® statistical package software v20.0 (IBM, USA) was used to statistically analyse the data. The *Kolmogorov–Smirnov* test was used to check continuous variables for normality. As non-Gaussians distributions were observed, the Mann–Whitney test was used for inferential analysis.

When analysing the specific fungi species, each species' proportions were generally categorized into tertiles. As in some cases the detected levels were so low, such as *Acremonium spp.*, the variable was dichotomized with the lower category consisting in values under the limit of detection and the higher category including those over the detection limit.

Microbial diversity score was defined as the sum of all the detected fungi groups/species. Due to the low number of observations with low and high diversity, the variable was categorized into quartiles, being the classrooms with lower diversity score in the first quartile, whereas the fourth quartile included those with higher diversity scores. Distributions of parameters by classroom, as well as diversity scores, are presented in Table S1.

Logistic regression adjusted for age and height was used to analyse the risk for allergic sensitization and asthma associated with the collected indoor air microbiology (total and specific values). Multinomial logistic regression was used for analysing the risk of inflammation reported by exhaled NO. The results were expressed as odds ratio (OR) and respective 95% confidence interval (95% CI).

Results

Classrooms with second tertile (OR = 1.26 [95% CI: 0.88–1.79]) and third tertile (OR = 1.40 [95% CI: 0.99–1.99]) concentrations of LPS showed a tendency for a higher risk of allergic sensitization (Fig. 2a). To further explore the possibility of LPS concentrations in classrooms being associated with the prevalence of allergic sensitization, nonparametric tests for independent samples were performed (Mann–Whitney test). The results showed that classrooms with higher concentrations of LPS had a significantly higher prevalence of allergic children (2.20 [0.89–4.25] vs. 2.60 [1.18–5.68], data presented as median [25–75%], respectively, non-sensitized vs sensitized, p = 0.02).

Diversity scores ranged from 1 to 11. Logistic regression models showed that there is a negative association between the number of fungi species in classrooms and the risk of allergic sensitization (Fig. 2b). These results were significant for the third (0.63 [95% CI: 0.40–0.98]) and fourth (0.60 [95% CI: 0.40–0.92]) quartile scores, which ranged from 7 to 8 (Table S1).

Additional pro-sensitization evidence was observed with specific fungal species, as classrooms with second tertile

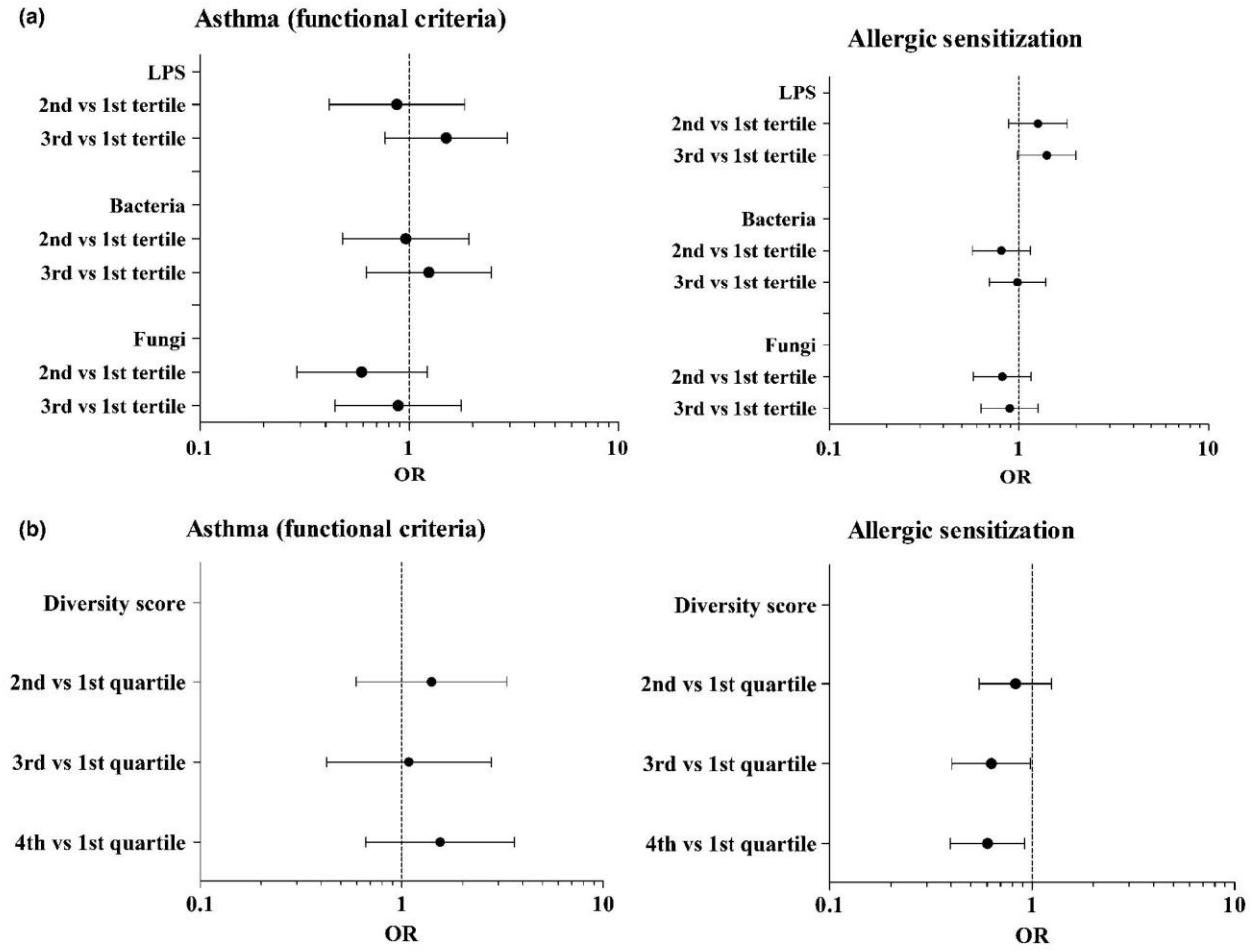

Figure 2 (a) Logistic regression between LPS, bacterial and fungal exposure in classrooms and the prevalence of asthma and atopy. (b) Logistic regression between fungal diversity scores and the prevalence of asthma and atopy. The results are expressed as odds ratios with 95% CI in logarithmic scale.

concentrations of *Penicillium spp* were associated with a higher risk of sensitization (OR = 1.46 [95% CI: 1.02–2.09]) and classrooms with third tertile concentrations showed an even higher risk (OR = 1.68 [95% CI: 1.18–2.40]). This tendency for a higher sensitization risk associated with *Penicillium spp* can be observed in Fig. 3. On the other hand, classrooms with higher concentrations of *Aspergillus fumigatus* (third vs first tertile, OR = 0.64 [95% CI: 0.47–0.87]), *Aspergillus niger* (detected vs not detected, OR = 0.62 [95% CI: 0.45–0.87]), *Chaetomium spp* (detected vs not detected, OR = 0.61 [95% CI: 0.39–0.96]) and *Rhizopus spp* (detected vs not detected, OR = 0.62 [95% CI: 0.45–0.87]) were associated with a lower risk of allergic sensitization (Fig. 3). Interestingly, the risk ratios were very similar between these species (children had 1.56–1.64 times less risk of being allergic).

To further explore the association of fungal diversity with the atopic syndrome, the risk of individuals with positive SPT suffering from allergic rhinitis and/or atopic dermatitis was also investigated. The results showed no significant differences between the quartiles of fungal diversity (Fig. S4).

No associations between LPS, bacteria or fungi concentrations in classrooms were found for any of the four definitions of asthma (Fig. S1). Also, no associations were observed between LPS, bacteria and fungi concentrations and the risk for having low or high exhaled NO (Fig. S2). The classrooms' specific fungal species and flora diversity were also not associated as a risk factor for any of the four different definitions of asthma (Fig. S3). Similar results were observed for exhaled NO values (data not shown).

Discussion

Our findings suggest that higher levels of endotoxin or *Penicillium* exposure in classrooms is associated with increased risk of allergic sensitization in school-aged children, while a higher fungal diversity showed a clear tendency for a decreased sensitization. However, similar tendencies were not observed when considering asthma or exhaled NO.

The main limitation of our study resides in its nature, which does not allow causal relationships to be established.

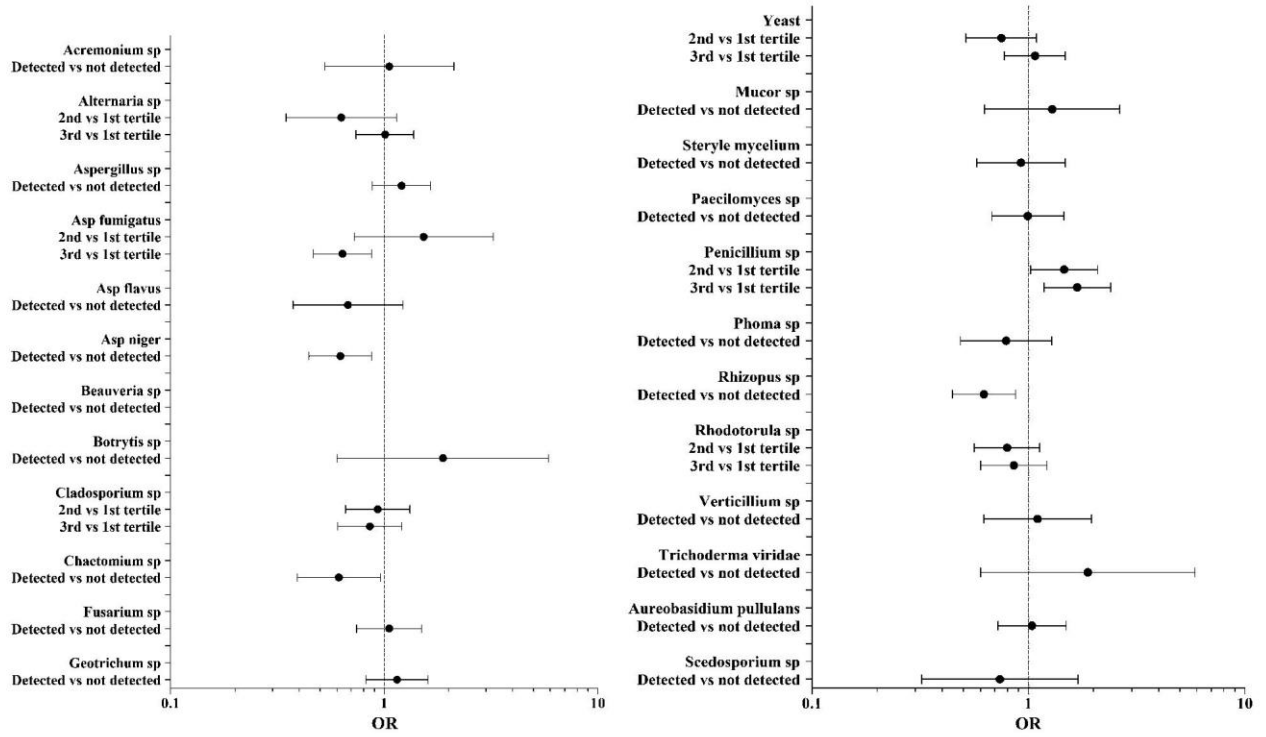

Figure 3 Logistic regression between exposure to specific fungi species in classrooms and the prevalence of atopy. The results are expressed as odds ratios with 95% CI in logarithmic scale.

Moreover, our observations consider only school-time exposure. While children spend most of their day at school, inside the classroom, they are still under exposure to microbiologic agents in other environments when they sleep, or on the playground, during recess; therefore, the complete exposome should be considered in future studies. Also, although in Portugal children usually stay in the same classroom through primary school, this may not be true in other countries, resulting in more exposure environments. Additionally, sensitization to one or more allergens does not necessarily imply a clinically relevant allergic status. Nevertheless, it was still possible to observe tendencies on how the prevalence of allergy and asthma in schoolchildren was affected by the exposure to indoor air bacteria and fungi in classrooms.

The strengths of this work reside in the substantial number of participants, either concerning classrooms or clinically analysed children. Moreover, the four distinct definitions of asthma prevent bias associated with the inclusion or exclusion of asymptomatic individuals and the diversity scores in the present study were calculated based on a large set of fungal species (24 in total), which enhances the impact of the results.

Endotoxin concentrations in classrooms were associated with a higher prevalence of sensitization to inhalant allergens in children, somehow corroborating the results obtained in the PASTURE study (20). These results need to be interpreted with caution, however, as children in the present study were considerably older and may have their immune systems further developed; thus, the impact of exposure to LPS in this age group may be substantially different than when exposition occurs in pre- and post-natal periods of life. This may also justify why, unlike in previous studies (5, 7, 9, 20, 21), exposure to environmental LPS did not show any significant association with asthma prevalence. A further matured immune system may also be responsible for the distinct results regarding exhaled NO associations with microbiologic parameters between the present work and the study performed by Casas et al. (22), which showed that exhaled NO at school age was lower in children exposed to LPS during the first 2–3 months of life.

Although no significant associations between exposure to total bacteria concentrations and allergic sensitization, asthma or exhaled nitric oxide levels were found, total fungi concentration is generally not a good predictor as it is not very specific and does not consider several confounding factors such as the species and diversity involved (1, 23). In this study, no fungi species in classrooms were associated with a lower prevalence of the four definitions of asthma. In contrast, the risk for sensitization was significantly lower in classrooms with higher concentrations of some *Aspergillus* species (along with *Chaetomium* and *Rhizopus* species), thus supporting the results reported by Ege et al. (5) within the GABRIELA study.

Classrooms with higher concentrations of *Penicillium spp* showed a significantly higher prevalence of allergic sensitization. This result corroborates those obtained by Sharpe et al. (24), which showed that *Penicillium* species were found in significantly higher concentrations in homes of individuals with

asthma. It is possible that this result is associated with the lower diversity scores in the respective classrooms, as the high concentrations of *Penicillium spp* may extensively reduce the sustainability of other fungi species. In contrast, classrooms with a higher fungal diversity and with lower concentrations of *Penicillium spp* were shown to be negatively associated with the prevalence of allergic sensitization, supporting the results from previous studies (25, 26). Despite having a clear impact on sensitization, fungal diversity was not a risk factor for allergic rhinitis and atopic dermatitis, suggesting that exposure to microbiologic agents may differently influence the atopic syndrome.

A different risk pattern for fungal diversity between the different definitions of asthma was also discernable, with the fungal diversity showing a tendency to be associated with a higher risk for lung function diagnosed asthma, but not for the other definitions. Sensitization odds ratio was lower than the unity for all tertiles, and a significant tendency for a lower risk of allergic sensitization was observed in classrooms with higher fungal diversity. This suggests that exposure to environmental fungi may have a completely different impact in asthma and allergy.

Part of these results are in line with recent advances in the biodiversity hypothesis (27), supporting the importance of exposure to a diverse biome to prevent development of allergic sensitization. Similarly to the results found by Ruokolainen et al. (28) in green areas around cities, microbiota biodiversity in classrooms seems to have an important role in the early immunomodulation. On the other hand, this study's results failed to support the evidence of beneficial exposure to bacterial endotoxins as observed in other studies concerning the biodiversity hypothesis (29). However, these are not isolated results. For instance, a domestic endotoxin exposure study in Cyprus also found higher exposure to endotoxins to be associated with increased allergic sensitization (30). It is possible that regional environmental and genotypic differences (for instance, Mediterranean versus Nordic regions) may have a role in this controversy. More region-wide studies are needed to further clarify the biodiversity hypothesis.

In conclusion, the microbiologic diversity in classrooms is associated with a lower risk of sensitization development in children, thus supporting the results from other studies concerning home exposure. However, this was not observed for the prevalence of asthma, suggesting that exposure to microbiologic agents may have a different mechanism of impact in both diseases. The four different asthma definitions used in this study further support this result. In face of these evidences, understanding the factors that influence diversity in the school environment may lead to public health recommendations for reducing the development of allergic sensitization or prevent symptomatic exacerbations in the future.

Acknowledgments

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References

1. Karvonen AM, Hyvarinen A, Rintala H, et al. Quantity and diversity of environmental microbial exposure and development of asthma: a birth cohort study. *Allergy* 2014: **69**: 1092–101.
2. Bush RK, Portnoy JM, Saxon A, Terr AI, Wood RA. The medical effects of mold exposure. *J Allergy Clin Immunol* 2006: **117**: 326–33.
3. Mendell MJ, Mirer AG, Cheung K, Tong M, Douwes J. Respiratory and allergic health effects of dampness, mold, and dampness-related agents: a review of the epidemiologic evidence. *Environ Health Perspect* 2011: **119**: 748–56.
4. Bornehag CG, Sundell J, Hagerhed-Engman L, Sigsggard T, Janson S, Aberg N. 'Dampness' at home and its association with airway, nose, and skin symptoms among 10,851 preschool children in Sweden: a cross-sectional study. *Indoor Air* 2005: **10**: 48–55.
5. Ege MJ, Mayer M, Normand A-C, et al. Exposure to environmental microorganisms and childhood asthma. *N Engl J Med* 2011: **364**: 701–9.
6. Gehring U, Heinrich J, Hoek G, et al. Bacteria and mould components in house dust and children's allergic sensitisation. *Eur Respir J* 2007: **29**: 1144–53.
7. Mendy A, Gasana J, Vieira ER, et al. Endotoxin exposure and childhood wheeze and asthma: a meta-analysis of observational studies. *J Asthma* 2011: **48**: 685–93.
8. Schram-Bijkerk D, Doekes G, Douwes J, et al. Bacterial and fungal agents in house dust and wheeze in children: the PARSIFAL study. *Clin Exp Allergy* 2005: **35**: 1272–8.
9. Braun-Fahrlander C, Riedler J, Herz U, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002: **347**: 869–77.
10. Bisgaard H, Li N, Bonnelykke K, et al. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. *J Allergy Clin Immunol* 2011: **128**: 646–52.
11. Morawska L, Afshari A, Bae GN, et al. Indoor aerosols: from personal exposure to risk assessment. *Indoor Air* 2013: **23**: 462–87.
12. Miller MR, Hankinson J, Brusasco V, et al. Standardisation of spirometry. *Eur Respir J* 2005: **26**: 319–38.
13. Dweik RA, Boggs PB, Erzurum SC, et al. An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications. *Am J Respir Crit Care Med* 2011: **184**: 602–15.
14. Williams HC, Burney PG, Pembroke AC, Hay RJ. The U.K. working party's diagnostic criteria for atopic dermatitis. III. Independent hospital validation. *Br J Dermatol* 1994: **131**: 406–16.
15. National Institute for Occupational Safety and Health. NIOSH manual of analytical methods 1998.
16. Standard E. CSN EN 13098: workplace atmosphere - guidelines for measurement of airborne micro-organisms and endotoxin. 2000.
17. Madureira J, Paciência I, Rufo JC, Pereira C, Teixeira JP, de Oliveira Fernandes E. Assessment and determinants of airborne bacterial and fungal concentrations in different indoor environments: Homes, child day-care centres, primary schools and

elderly care centres. *Atmos Environ* 2015: **109**: 139–46.
18. Madureira J, Pereira C, Paciência I, Teixeira JP, de Oliveira Fernandes E. Identification and levels of airborne fungi in Portuguese primary schools. *J Toxicol Environ Health A* 2014: **77**: 816–26.
19. International Organization for Standardization. 4833-1: microbiology of the food chain – horizontal method for the enumeration of microorganisms – part 1: colony count at 30 degrees C by the pour plate technique. 2013.
20. Karvonen AM, Hyvarinen A, Gehring U, et al. Exposure to microbial agents in house dust and wheezing, atopic dermatitis and atopic sensitization in early childhood: a birth cohort study in rural areas. *Clin Exp Allergy* 2012: **42**: 1246–56.
21. Thorne PS, Kulhankova K, Yin M, Cohn R, Arbes SJ Jr, Zeldin DC. Endotoxin exposure is a risk factor for asthma: the national survey of endotoxin in United States housing. *Am J Respir Crit Care Med* 2005: **172**: 1371–7.
22. Casas L, Tischer C, Wouters IM, et al. Early life microbial exposure and fractional exhaled nitric oxide in school-age children: a prospective birth cohort study. *Environ Health* 2013: **12**: 12–103.
23. Newson R, Strachan D, Corden J, Millington W. Fungal and other spore counts as predictors of admissions for asthma in the Trent region. *Occup Environ Med* 2000: **57**: 786–92.
24. Sharpe RA, Bearman N, Thornton CR, Husk K, Osborne NJ. Indoor fungal diversity and asthma: a meta-analysis and systematic review of risk factors. *J Allergy Clin Immunol* 2015: **135**: 110–22.
25. Behbod B, Sordillo JE, Hoffman EB, et al. Asthma and allergy development: contrasting influences of yeasts and other fungal exposures. *Clin Exp Allergy* 2015: **45**: 154–63.
26. Dannemiller KC, Mendell MJ, Macher JM, et al. Next-generation DNA sequencing reveals that low fungal diversity in house dust is associated with childhood asthma development. *Indoor Air* 2014: **24**: 236–47.
27. Haahtela T. What is needed for allergic children? *Pediatr Allergy Immunol* 2014: **25**: 21–4.
28. Ruokolainen L, von Hertzen L, Fyhrquist N, et al. Green areas around homes reduce atopic sensitization in children. *Allergy* 2015: **70**: 195–202.
29. Carlsten C, Ferguson A, Dimich-Ward H, et al. Association between endotoxin and mite allergen exposure with asthma and specific sensitization at age 7 in high-risk children. *Pediatr Allergy Immunol* 2011: **22**: 320–6.
30. Nicolaou N, Yiallouros P, Pipis S, Ioannou P, Simpson A, Custovic A. Domestic allergen and endotoxin exposure and allergic sensitization in Cyprus. *Pediatr Allergy Immunol* 2006: **17**: 17–21.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Logistic regression between LPS, bacterial and fungal exposure in classrooms and the prevalence of asthma (considering the four different classification methods). The results are expressed as odds ratios with 95% CI in logarithmic scale.

Figure S2. Logistic regression between LPS, bacterial and fungal exposure in classrooms and the levels of FeNO. The results are expressed as odds ratios with 95% CI in logarithmic scale.

Figure S3. Logistic regression between fungal diversity scores in classrooms and the prevalence of asthma (considering the four different classification methods). The results are expressed as odds ratios with 95% CI in logarithmic scale.

Figure S4. Logistic regression between fungal diversity scores in classrooms and the prevalence of allergic disease (positive skin-prick tests with reported allergic rhinitis and/or atopic dermatitis). The results are expressed as odds ratios with 95% CI in logarithmic scale.

Table S1. Concentration of microbial agents and distribution of fungal species measured in the participating classrooms.

Study II

J. Cavaleiro Rufo, I. Paciência, D. Silva, C. Martins, J. Madureira, E. Oliveira Fernandes, P. Padrão, P. Moreira, L. Delgado, A. Moreira.

Swimming pool exposure is associated with autonomic changes and increased airway reactivity to a beta-2 agonist in school aged children: A cross-sectional survey.

PLoS ONE. 2018. 13(3): e0193848.

PLOS ONE

RESEARCH ARTICLE

Swimming pool exposure is associated with autonomic changes and increased airway reactivity to a beta-2 agonist in school aged children: A cross-sectional survey

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Abstract

Background

Endurance swimming exercises coupled to disinfection by-products exposure has been associated with increased airways dysfunction and neurogenic inflammation in elite swimmers. However, the impact of swimming pool exposure at a recreational level on autonomic activity has never been explored. Therefore, this study aimed to investigate how swimming pool attendance is influencing lung and autonomic function in school-aged children.

Methods

A total of 858 children enrolled a cross sectional survey. Spirometry and airway reversibility to beta-2 agonist, skin-prick-tests and exhaled nitric oxide measurements were performed. Pupillometry was used to evaluate autonomic nervous function. Children were classified as current swimmers (CS), past swimmers (PS) and non-swimmers (NS), according to the amount of swimming practice.

Results

Current swimmers group had significantly lower maximum and average pupil constriction velocities when compared to both PS and NS groups (3.8 and 5.1 vs 3.9 and 5.3 vs 4.0 and 5.4 mm/s, $p = 0.03$ and $p = 0.01$, respectively). Moreover, affinity to the beta-2 agonist and levels of exhaled nitric oxide were significantly higher in CS when compared to NS (70 vs 60 mL and 12 vs 10 ppb, $p<0.01$ and $p = 0.03$, respectively). A non-significant trend for a higher risk of asthma, atopic eczema and allergic rhinitis was found with more years of swimming practice, particularly in atopic individuals (β = 1.12, 1.40 and 1.31, respectively). After case-

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case analysis, it was possible to observe that results were not influenced by the inclusion of individuals with asthma.

Conclusions

Concluding, swimming pool attendance appears to be associated with autonomic changes and increased baseline airway smooth muscle constriction even in children without asthma.

Introduction

Swimming is not only one of the most practiced sports worldwide, but is frequently recommended by physicians, due to the multiple associated benefits. These recommendations often target patients with allergic and respiratory diseases since it is believed that health benefits of indoor swimming significantly surpass the risks associated with the practice, such as drowning or infections [1].

However, the endurance swimming exercises coupled to disinfection by-products exposure has been shown responsible for increased airways dysfunction in elite swimmers [2–7]. Moreover, airways dysfunction, such as airway hyperresponsiveness and bronchoconstriction, has been associated with parasympathetic dysautonomia in athletes, including swimmers [8–10]. Shortly, airways are innervated by postganglionic parasympathetic-cholinergic nerves which, when activated, are capable of reducing the lumen of small bronchi and bronchioles, significantly increasing airways resistance. It is therefore comprehensible that if exogenous stimuli may influence the parasympathetic tonus, they may also indirectly affect airway smooth muscle constriction [11].

Swimming pool exposure is known to be responsible for airway dysfunction in swimmers due to chlorine-based disinfection by-products [5, 12, 13]. These products may be ingested, inhaled or absorbed via skin during the swimming practice, thus resulting in airways epithelium damage and eventually airway hyperresponsiveness [4, 14]. This has been emphasized by several studies published in the last decade, showing an association between swimming pool attendance and a higher risk of asthma in children [15–17], which has lead the scientific community to question the beliefs regarding swimming practice benefits in children [18]. Nevertheless, endurance exercises and extensive training volume are frequently held responsible for the aforementioned autonomic nervous function changes, somehow disregarding the possible influence of the indoor swimming pool environment on the parasympathetic tonus. In fact, environmental pollutants are known to promote a higher expression of transient receptor potential vanilloid 1 [19–21], which is the centre of almost all neuronal inflammatory signalling pathways [22]. However, the impact of swimming pool exposure on autonomic activity has never been explored. It is therefore possible that exposure in swimming pools is also associated with dysautonomia in swimmers, independently of the training volume.

To further explore this hypothesis, the present study aimed to investigate how swimming pool attendance may relate to airway constriction and dysautonomia in school-aged children.

Methods

Participants and study design

A cross-sectional survey of children attending the 3rd and 4th grades from 20 primary schools in Porto, Portugal, was conducted from January 2014 to March 2015. The study was authorized

by the Ethics Committee for Health of S. João Hospital Centre and by the schools' Directive Councils. Parents and legal guardians of 1602 children attending the participating schools were contacted and received written information concerning the project. A written consent was retrieved for 916 children (57.2% participation rate), but only 858 (aged 7 to 12 years old) completed the clinical assessment as the remaining 58 children refused to perform some of the clinical tests despite the legal guardians' consent. Height, weight, lung function (spirometry with bronchodilation) and exhaled nitric oxide (NO) levels were measured in participating children. Skin-prick-tests (SPT) and pupillometry were also performed on the participants by a trained professional. A standardized ISAAC-based self-reported questionnaire focused on their child's respiratory and allergic symptoms, as well as on swimming practice, was filled by the parents.

Physiological assessments

Children's lung function and airway reversibility were assessed according to the ATS/ERS guidelines [23]. Lung function data was retrieved before and 15 minutes after bronchodilation, which was stimulated by administering an inhaled beta-2 agonist (400 μg of inhaled salbutamol).

Eosinophilic airway inflammation was assessed by measuring exhaled NO levels using the NObreath (Bedfont Scientific Ltd. UK). The results were expressed as parts per billion (ppb) and stratified according to the official ATS guidelines for children [24].

Allergic sensitization was evaluated by SPT on their forearm using a QuickTest™ applicator and extracts of *Dermatophagoides pteronyssinus*, weed pollen mix, grass pollen mix, cat dander, dog dander and *Alternaria alternata*, negative control (extracts dilutant), and a positive control (histamine at 10mg/mL), all belonging to the same batches (Hall Allergy, Netherlands). Results were read 20 minutes afterwards. If children were on antihistamines or topical corticosteroids on the skin within the previous 7 days, SPTs were postponed.

Regarding pupillometry, the participants spent 15 minutes in a semi-dark and quiet room to allow their eyes to adjust to the low lighting levels before measurement. Pupillary measurements were taken with the portable infrared PLR-200™ Pupillometer (NeurOptics Inc, CA, USA). The complete pupillometry methodology has been thoroughly described in previous publications [8, 25]. Shortly, the pupil constriction response to a light stimulus represents parasympathetic activity, and the dilatation represents sympathetic activity. The following parameters were recorded: percentage of pupil constriction (CON); average and the maximum constriction velocities (ACV and MCV, respectively); minimum and maximum pupil diameter; average dilation velocity (ADV); and the time in seconds at 75% recovery of pupil size (T75). Since there was no side-to-side difference in pupil responses, all pupillary data reported in the results was obtained from the right eye, in a similar approach to Muppidi *et al.* [26]. If a valid measurement was not obtainable, measurements from the left eye were used instead.

Definition of clinical and exposure outcomes

To improve observational power on asthma prevalence, four different operational asthma definitions were adopted: i) *Clinical criteria*–at least a 12% and over 200mL increase in FEV_1 after bronchodilation and/or self-report of asthma diagnosed by a physician with reported symptoms (wheezing, dyspnoea or dry cough) occurring in the past 12 months; ii) *Functional criteria*–at least a 12% and over 200mL increase in FEV_1 after bronchodilation; iii) *Treated asthma*–self-report of asthma diagnosed by a physician and currently under inhaled corticosteroid treatment; and iv) *Ever asthma*–self-report of asthma diagnosed by a physician.

Allergic sensitization was defined by a positive SPT to at least one of the tested allergens (wheal > 3mm) coupled to a positive histamine response (wheal > 3mm) and no positivity in the negative control (wheal < 3mm) [27].

Atopic eczema was defined as a positive answer to the question "Did your child ever had itchy skin alterations that appeared and disappeared for at least 6 months, during the past 12 months?" followed by a positive answer to "Did these skin alterations ever affected elbow and knee joints, ankles, between thighs, or around the neck, ears or eyes?", based on the UK Working Party diagnostic criteria for the definition on atopic eczema [28]. Otitis definition was based on a positive answer to the question "Did your child had ear pain or otitis in the last 12 months not associated with a cold or a flu?", while allergic rhinitis was defined as a positive answer to the question "Did your child suffered from recurrent sneezing, runny nose or nasal congestion, in the past 12 months, not associated with a cold or a flue?".

Through questionnaires, subjects were defined as *current swimmers* (CS) if parents answered "Yes" to the question: "Does your child swim frequently in an indoor swimming pool (at least once per week)?"; if they answered "No, but he/she used to", participants were classified as *past swimmers* (PS); otherwise, if they answered "No", they were regarded as *non-swimmers* (NS). For the CS and PS groups, cumulative swimming pool exposure, expressed in years, was calculated. Due to incomplete or unanswered questionnaires, 83 participants were excluded from the study. Therefore, a total of 205, 228 and 342 children were classified as CS, PS and NS, respectively. The question "Does your child partake in any sport activity outside of normal school-period, at least once per week?" was used to scrutinize if non or past swimmers practiced any other type of sport, in order to exclude any conceivable bias associated with sedentarism or training volume. Fig 1 illustrates the recruitment flow and group selection criteria whereas Table 1 summarizes health assessments per participant group.

Statistical analysis

The SPSS® statistical package software v20.0 (IBM, USA) was used to statistically analyse the data. The Kolmogorov-Smirnov test was used to check continuous variables for normality. Whenever non-Gaussians distributions were observed, non-parametric tests were used for inferential analysis.

T-student test and one-way ANOVA (for normal distributions) or Mann-Whitney and Kruskal-Wallis tests (for non-parametric distributions) were used to compare continuous variables between two or more than two groups of individuals, respectively. Significant differences were reported with an α-value inferior to 5% ($p<0.05$).

The Spearman's correlation test was used to find correlations between the number of years in swimming practice and the measurable outcomes. Risk analysis was performed to identify the risk of allergic disease development associated with early swimming and logistic regression analysis was then used to find associations between the number of years in swimming practice and the binary categorical variables. Results were expressed in OR[95%CI] and β[95%CI], respectively. Finally, multinomial logistic regression was used to compare ranked terciles of years in swimming practice to further evaluate the influence of cumulative swimming pool attendance. The results were reported as OR[95%CI].

Results

Children in the CS group had significantly lower maximum (MCV) and average pupil constriction velocities (ACV) when compared to both PS and NS groups, MCV (mm/s, mean±sd): 5.1±1.0 *vs* 5.3 ±1.0 *vs* 5.4 ±0.9, respectively ($p = 0.010$); and ACV(mm/s, mean±sd): 3.8 ±0.7 *vs* 3.9 ±0.8 *vs* 4.0 ±0.7, respectively ($p = 0.030$). Moreover, levels of exhaled NO and changes in

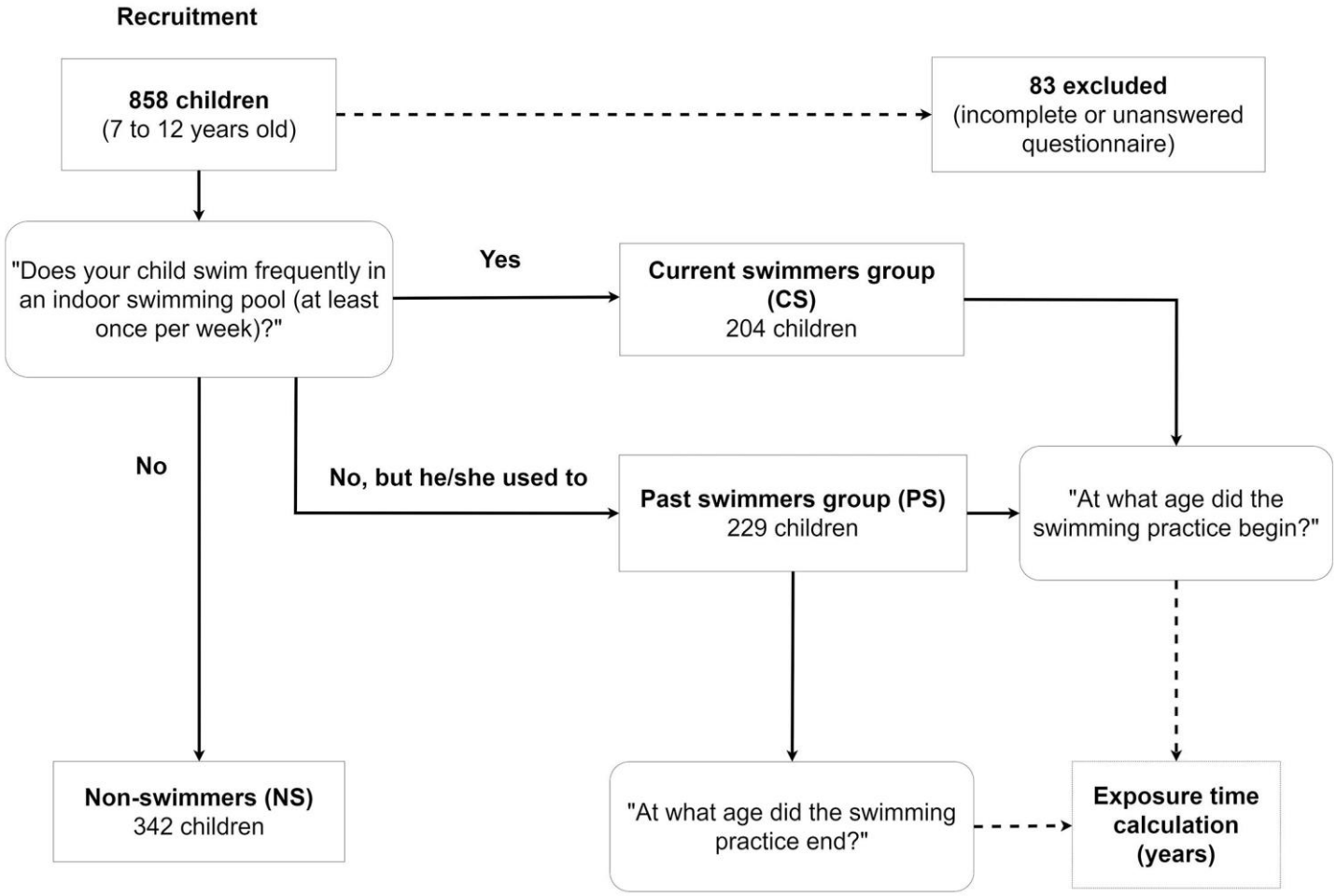

Fig 1. Flow chart of the recruited participants. Round-edged boxes represent the questions that allowed classification of participants into three different groups according to the respective answers: current, past and non-swimmers.

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airway reversibility volume after administration of a beta-2 agonist were significantly higher in the CS group when compared to NS (respectively, median [P25 to P75]: 12ppb [7 to 20] *vs* 10 ppb [5 to 19], $p = 0.030$; and 70mL [20 to 130] vs 60mL [-10 to 110], $p = 0.007$) (Table 1 and Fig 2). These results were not influenced by sedentarism and/or other sport practice since no significant difference was found between percentage of weekly sport practisers between groups ($p = 0.965$).

There were no differences between the three groups of participants for the prevalence of asthma (for any of the 4 definitions) or allergic sensitization, although a significantly higher occurrence of otitis was observed in NS when compared to PS (32.4 *vs* 21.0%, respectively; $p = 0.013$), but not to CS (32.4 *vs* 27.0%, respectively; $p = 0.251$).

Differences found in the studied physiological outcomes were not influenced by the inclusion of individuals with asthma in the groups since, with the exception of atopic eczema, there were no significant changes when exclusively comparing individuals with clinical defined asthma (S1 Table).

As expected, the number of years in swimming practice was significantly higher in CS when compared to PS (mean (±SD) = 3.9 (±2.2) vs 2.4 (±1.6), respectively; $p<0.010$). To investigate the effect of cumulative exposure resultant from swimming practice, correlations

Table 1. Characteristics of the participants.

	Current swimmers	Past swimmers	Non-swimmers	*p*
N (males)	205 (99)	228 (115)	342 (175)	- -
Age (years, mean ± sd)	8.7 ±0.8	8.6 ±0.7	8.9 ±0.8	**0.011**[¥]
Weight (kg)	30.9 (26.6 to 36.9)	32.1 (28.2 to 37.8)	30.8 (26.9 to 37.3)	**0.048**
Height (cm)	135 (130 to 139)	136 (131 to 141)	136 (131 to 141)	0.196
Sport practisers (%)	100.0	98.3	98.2	0.965
Lung function				
FVC (L)	1.88 (1.71 to 2.15)	1.91 (1.71 to 2.18)	1.88 (1.66 to 2.10)	0.125
FEV_1 (L)	1.73 (1.58 to 1.95)	1.78 (1.60 to 1.99)	1.74 (1.55 to 1.92)	0.081
FEV_1/FVC (%)	92.8 (89.1 to 96.1)	92.5 (89.0 to 96.6)	92.7 (88.8 to 96.4)	0.998
FEF_{25-75} (L/s)	2.23 (1.97 to 2.71)	2.36 (1.93 to 2.71)	2.27 (1.91 to 2.59)	0.423
PEF (L/s)	3.77 (3.30 to 4.38)	3.77 (3.38 to 4.21)	3.69 (3.27 to 4.27)	0.641
FEV_1 reversibility (mL)	70 (20 to 130)	60 (10 to 120)	60 (-10 to 110)	**0.028**
FEV_1 reversibility (%)	4.0 (1.3 to 7.2)	3.4 (0.6 to 6.7)	3.1 (-0.7 to 6.2)	**0.040**
FVC reversibility (mL)	40 (-30 to 100)	30 (-30 to 80)	20 (-40 to 90)	0.219
Exhaled NO (ppb)	12 (7 to 20)	11 (6 to 20)	10 (5 to 19)	0.086
Asthma[β]				
Clinical criteria (n, %)	11.7%	8.3%	9.4%	0.459[*]
Functional criteria (n, %)	6.3%	7.0%	6.4%	0.957[*]
Treated asthma (n, %)	6.3%	4.0%	6.1%	0.441[*]
Ever asthma (n, %)	7.3%	4.8%	7.3%	0.438[*]
Atopic eczema (n, %)	66.7%	62.9%	54.4%	0.465[*]
Allergic rhinitis (n, %)	33.3%	33.8%	31.1%	0.907[*]
Allergic sensitization (%)	32.8	39.5	34.2	0.302[*]
Otitis (n, %)	27.0%	21.0%	32.4%	**0.043**[*]
Pupillometry				
Maximum (mm, mean ± sd)	5.2 ±0.9	5.3 ±1.0	5.3 ±0.8	0.278[¥]
Minimum (mm, mean ± sd)	3.4 ±0.6	3.4 ± 0.6	3.4 ±0.6	0.537[¥]
CON (%, mean ± sd)	35 ±5	36 ±5	36 ±5	0.203[¥]
ACV (mm/s, mean ± sd)	3.8 ±0.7	3.9 ±0.8	4.0 ±0.7	**0.030**[¥]
MCV (mm/s, mean ± sd)	5.1 ±1.0	5.3 ±1.0	5.4 ±0.9	**0.010**[¥]
ADV (mm/s, mean ± sd)	1.2 ±0.3	1.2 ±0.4	1.2 ±0.3	0.709[¥]
T75 (s, mean ± sd)	1.7 ±0.7	1.7 ±0.7	1.7 ±0.7	0.987[¥]

Data reported as median (P25 to P75) unless otherwise stated. BMI: body mass index; FEV_1: forced expiratory volume in the first second of FVC; PEF: Peek expiratory flow; FVC: forced vital capacity; FEF_{25-75}: forced expiratory flow middle portion of FVC; EBC: exhaled breath condensate; CON: percentage of pupil constriction; ACV: average constriction velocity; MCV: maximum conscription velocity; ADV: average dilation velocity. The p values signalling differences between the three groups were calculated using the Kruskal-Wallis test for non-parametric variables, with the exception of cases marked with (*) which were calculated using qui-square tests, and (¥), which were calculated using one-way ANOVA (for normal distributions).

[β]The following operational asthma definitions were adopted: i) Clinical criteria–at least a 12% increase in FEV1 after bronchodilation and over 200mL and/or asthma diagnosed by a physician with reported symptoms (wheezing, dyspnoea or dry cough) occurring in the past 12 months; ii) Functional criteria–at least a 12% increase in FEV1 after bronchodilation and over 200mL; iii) Treated asthma criteria–asthma diagnosed by a physician and currently under inhaled corticosteroid treatment; and iv) Ever asthma–asthma diagnosed by a physician.

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between the number of years in swimming practice and continuous variables of the measured health outcomes were calculated using the Spearman's correlation test (Table 2). Significant correlations were observed for baseline FEV_1 (rho = 0.11), PEF (rho = 0.18), and the CON (rho = 0.12), ADV (rho = -0.13) and T75 (rho = 0.19) parameters of pupillometry.

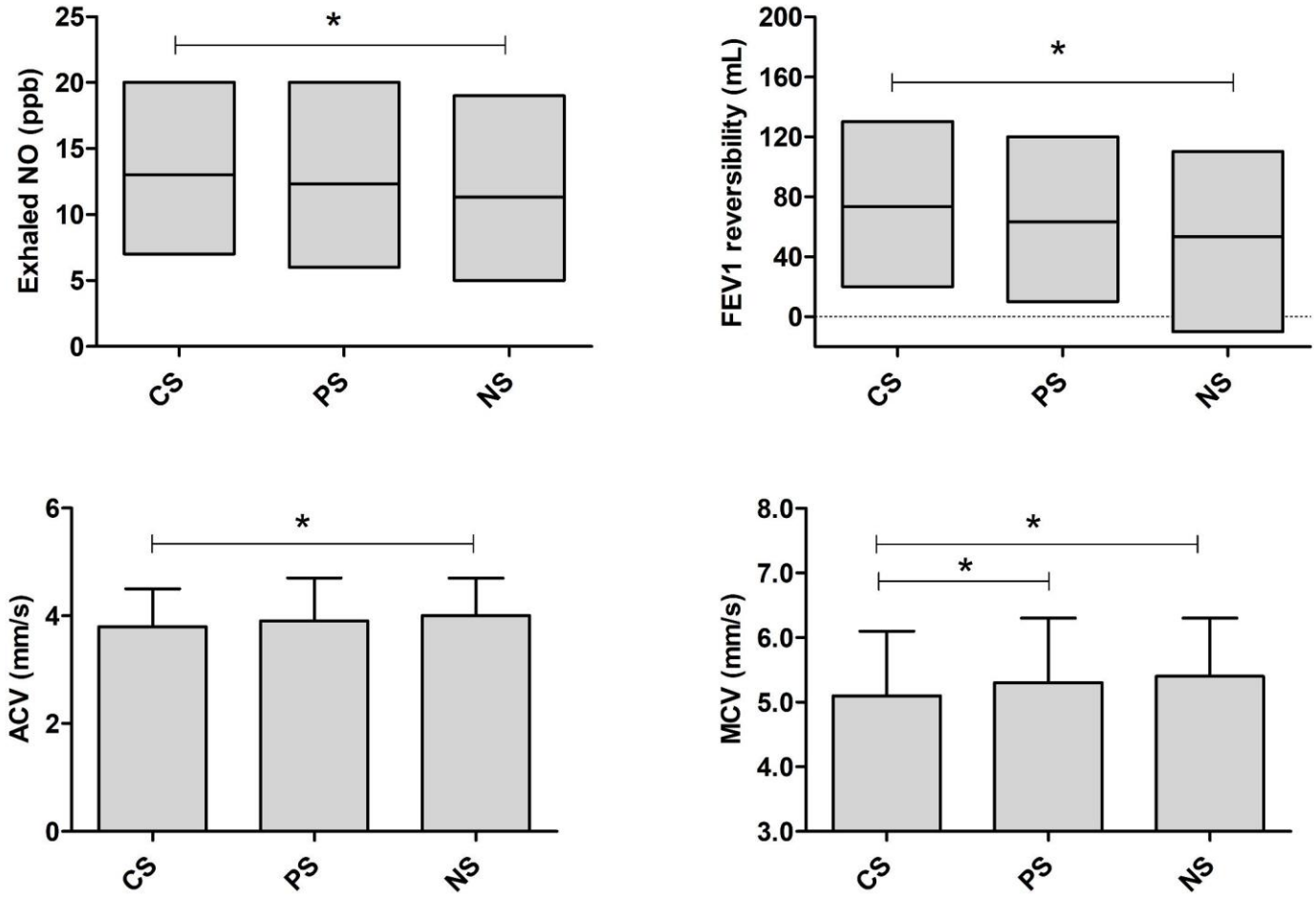

Fig 2. Median (with 25 and 75 percentiles) levels of exhaled NO and FEV1 reversibility, and mean ±SD of measured average constriction velocity (ACV) and maximum constriction velocity (MCV) among the three groups. CS–current swimmers; PS–past swimmers; NS–non-swimmers. *Represents significant differences between two groups indicated by the extremities of the horizontal line ($p<0.05$).

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Early swimming, which was defined as individuals who started swimming before the age of 3, based on a previous study design [29], was associated with a higher tendency for allergic disease development, with a significantly higher risk of asthma defined by the functional criterion (Fig 3). Furthermore, the logistic regression analysis showed a non-significant trend for a higher risk of asthma by lung function criteria (β[95%CI] = Atopic: 1.12[0.81 to 1.55]; Non-atopic: 1.17[0.92 to 1.55]), atopic eczema (β[95%CI] = Atopic: 1.40[0.87 to 2.24]; Non-atopic: 1.00[0.70 to 1.43]) and allergic rhinitis (β[95%CI] = Atopic: 1.31[0.90 to 1.90]) with more years of swimming practice (Fig 4). These results were replicated by the multinomial logistic regression, where a higher risk of asthma (functional criteria), allergic rhinitis and atopic eczema were observed within the highest tercile of years in swimming practice (S1 and S2 Tables).

Discussion

Our findings provide support to the hypothesis that swimming pool attendance is associated with autonomic changes even in non-elite swimmers. Firstly, we observed by pupillometry that swimming pool exposure in school-aged children is associated with parasympathetic dysautonomia. Secondly, a higher volume of airway reversibility in response to an inhaled beta-2

Table 2. Spearman's correlation test between continuous clinical parameters and the number of years in swimming practice. Values represent the Spearman's correlation coefficient. Significant correlations are expressed in bold.

	Number of years in swimming practice (rho)
Lung function parameters	
FVC	.098
FEV_1	**.111**
FEV1/FVC	-.001
FEF 25–75%	.054
PEF	**.184**
Forced expiratory flow	.008
FEV1 reversibility	.064
Exhaled NO	.016
Pupillometry parameters	
Maximum diameter	.014
Minimum diameter	-.030
CON	**.118**
ACV	.013
MCV	-.001
ADV	**-.126**
T75	**.186**

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agonist, suggestive of increased baseline airway smooth muscle constriction, was also observed in children that frequently attend swimming pools. Thirdly, these subtle changes appear to be reversible with swimming practice cessation, as no evidence of parasympathetic perturbances or airway constriction were found in children that used to swim in the past, even if they kept practicing any other type of sport.

As with all proof of concept studies, this one has its limitations. The cross-sectional nature does not allow causal relationships to be established. Nevertheless, the inclusion of a group of participants that were past swimmers may allow the estimation of persistence changes associated with indoor swimming exposure. Another limitation may be inherent to the question used to allocate participants to each group of swimming exposure, since although being "yes or no" questions, they are not exempt of reporting bias. Regarding cumulative exposure, no data on number of hours of training per week has been collected. However, considering the age range of participants, rarely should the practice represent more than 2 hours per week of active swimming. Although continuous outdoor swimming practice is not commonly performed by Portuguese children outside the summer season, information regarding the swimming pool environment has not been collected which may be seen as a limitation. Moreover, knowing the family atopic background could also help to determine if some individuals were already pre-disposed to develop some of these changes. We also cannot exclude reverse causation bias as the number of children with otitis was lower in past swimmers. Nevertheless, our study has several unique strengths. It is community based, not affected by a swimming pool recruitment strategy bias. Moreover, the exclusion of sedentarism-based bias, coupled to the high number of participants per group, as well as the extensive workup evaluation, particularly for assessing the autonomic nervous system and airway physiology, increases the robustness of the findings. Lastly, pupillometry is a sensible method that allows the detection of subtle changes in the oculosympathetic pathways, as previously demonstrated by Yoo *et al* (2017) with the Horner's eye syndrome [30].

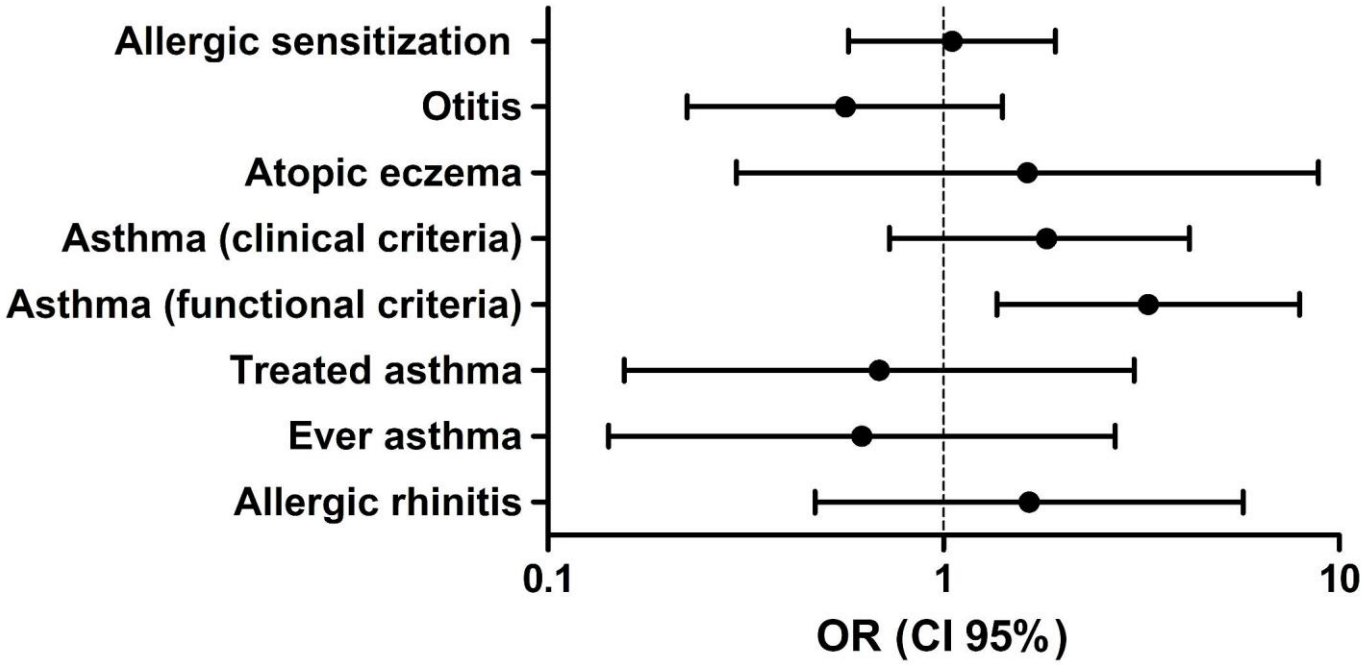

Fig 3. Risk analysis of early swimming (children who started swimming before the age of 3) in the development of allergic diseases and asthma. Results are represented as OR (5–95%CI).

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Neurogenic inflammation has been suggested to contribute to the recognized higher prevalence of asthma in athletes training and competing in environments with a high airway irritation potential [31]. Transient receptor potential vanilloid 1 is the centre of almost all neuronal inflammatory signalling pathways; this ion-channel is often co-localized with sensory neuropeptides in the same axon of a primary neuron and its stimulation can lead to the release of these substances. It is expressed in primary sensory neurons, pulmonary smooth muscle cells, bronchial and tracheal epithelial cells and dendritic cells in the lung [22]. Known physical activators of these channels include noxious temperature such as heat or cold, changes in membrane potential, mechanical or osmotic stress, and arachidonic acid metabolites [32]. A recent

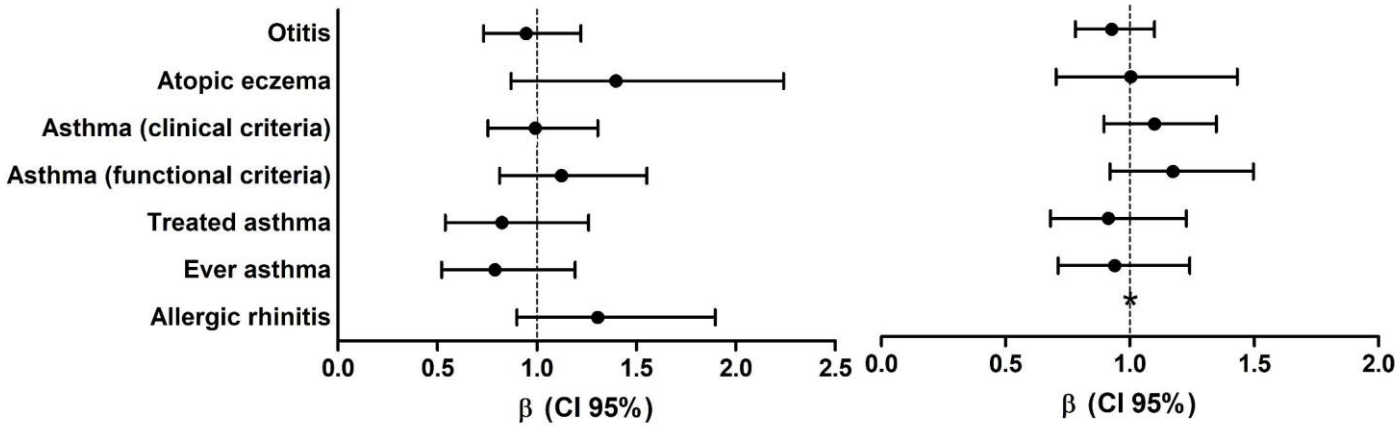

Fig 4. Logistic regression between the number of years in swimming practice and the assessed clinical outcomes (adjusted for age and "early swimming"). Participants were stratified according to their atopic status: A) Atopic children; and B) Non-atopic children. Results are represented as β (5–95%CI). *There were no non-atopic children reporting symptoms of allergic rhinitis.

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study showed increased levels of substance P in sputum of competitive swimmers suggesting it may be the result of a compensatory response to the sympathetic stimulation promoted by intensive training, neurogenic inflammatory response to swim stress and/or a local airway chemosensory reflex to chlorine by-products exposure in swimmers [33]. However, altered parasympathetic tonus in healthy swimmers has until this moment been almost exclusively associated with endurance training. Although the high training volumes may certainly influence the autonomic nervous function, this study results now show that parasympathetic dysfunction does occur in healthy children swimmers not undergoing endurance training. Our findings further extend this observation suggesting that autonomic changes may not only be caused by high training volumes, but also by environmental exposure, even in young children. The significantly higher exhaled NO levels observed in swimmers, although subtle and within physiologic reference values, support the co-existence of airways inflammation in those children. Therefore, it is possible that swimming pool exposure may be responsible for airway constriction at two levels: directly, by inhalation of disinfection by-products which will damage airway epithelium and increase oxidative stress [5]; and indirectly, by causing parasympathetic dysautonomia which may lead to a reflex vasoconstriction of bronchial venules, reducing the size of the bronchial lumen and generating increased airways resistance [34].

In the present study, the subtle changes associated with swimming pool attendance tended to disappear in children who discontinued swimming practice, since autonomic parameters, lung function and exhaled NO levels were not significantly different between past and non-swimmers. These results, coupled to the absence of significant correlations between the number of years in swimming practice and the airway reversibility or exhaled NO, suggests that swimming pool environment was the main responsible for the changes in airway inflammation biomarkers, which tend to disappear after ceasing the practice. In addition, parasympathetic activity also appears to be re-established after swimming cessation, since only sympathetic parameters seem to be significantly correlated with the number of years in swimming practice. Although this changes in sympathetic activity may be associated with more years of regular physical exercise [35], this cannot be concluded in the present study since only swimming practice was considered and children may have performed other physical activities at the same time period. Interestingly, these "reversibility" results support the hypothesis presented by Lomax (2016) regarding airway dysfunction in elite swimmers [36]. By systematically reviewing relevant publications, Lomax hypothesised that chlorine exposure in swimming pools coupled to endurance swimming exercises caused epithelial damage that could lead to several airway symptoms, including bronchoconstriction [37], but airway epithelium was estimated to be replenished every 30 to 50 days in the absence of continued damage [36, 38]. When under continuous exposure, the injury-repair process of the airways epithelium may lead to respiratory disorders and, eventually, airway hyperresponsiveness [36, 37, 39]. While children in the present study were certainly not submitted to endurance training, they were still exposed to chlorine-based disinfection by-products.

Although not necessarily a novelty, results also showed that children that started the swimming practice before the age of 3 were tendentially under a higher risk for asthma and allergic disease development, thus supporting the hypothesis presented by Voisin and co-workers in 2014 [29]. This suggests that exposure to a highly chlorinated environment during the early years of life may be even more prejudicial, which further underlines the importance of the practisers' susceptibility. Nevertheless, and as observed for otitis, reverse-causality bias cannot be excluded and results should be interpreted with caution.

It is important to notice that several positive traits are associated with swimming pool attendance. In line with other population-based studies [1, 40–43], the results showed that swimming pool attendance was not associated with increased of allergic sensitization in children,

although a trend for a higher risk of asthma (functional criteria), atopic eczema and rhinitis, was observed in swimmers. Swimming practice was also not associated with a higher prevalence of allergic sensitization and no adverse effects on baseline lung function parameters were observed. In fact, this study shows that children with more years in swimming practice generally have improved baseline lung function, supporting several other studies in the last two decades [44, 45], including those focused in prepubertal children, such as the one published by Courteix *et al* in 1997 [46]. However, seldom has exercise-induced bronchoconstriction been scrutinized in these studies and, as observable in the present study, children that attend swimming pools have a significantly higher exhaled NO and reversibility of FEV_1. These results, coupled to the altered parasympathetic function in the current swimmers group suggests that exposure during indoor swimming may contribute to airways constriction independently of the training volume and asthma or atopic status.

Two important aspects of the present study need to be taken into consideration: first, the airway reversibility and eosinophilic airway inflammation observed in active swimmers group are non-pathological according to current guidelines [24, 27]; and second, the measured parameters appear to normalize after the practice cessation, as observed with the children in the PS group, independently of continuing other sportive activities. While the physiological mechanisms of the association between altered autonomic function and environmental exposure have not been fully explained, there is evidence showing that traffic-related air pollution may be responsible for disturbances of the autonomic system [47]. Baja *et al* (2013), using structural equation models, also observed that traffic pollution may decrease parasympathetic tone among diabetic elderly [48]. Therefore, we may assume the observed autonomic changes could be associated with indoor swimming pool attendance in susceptible individuals, such as schoolchildren.

Conclusion

Concluding, swimming pool attendance appears to be associated with autonomic changes even in non-elite swimmers, such as children. Continued swimming pool exposure in school-aged children may cause parasympathetic dysautonomia and increased response to inhaled beta-2 agonists, consequently resulting in increased baseline airway smooth muscle constriction. Although these subtle changes appear to be reversible with swimming practice cessation, it is interesting to note they seem to mirror, in a lower scale, those that characterise swimmers' asthma: parasympathetic dysautonomia and bronchoconstriction with a neurogenic inflammation component related to high ventilation rates.

Supporting information

S1 Table. Clinical parameters of individuals with asthma, between the three groups.
(DOCX)

S2 Table. Crude risk analysis between the terciles of years in swimming practice and the development of allergic diseases and asthma.
(DOCX)

S3 Table. Adjusted risk analysis between the terciles of years in swimming practice and the development of allergic diseases and asthma.
(DOCX)

S1 Dataset. Database containing the relevant data variables for the study analysis.
(SAV)

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References

1. Font-Ribera L, Villanueva CM, Gracia-Lavedan E, Borras-Santos A, Kogevinas M, Zock JP. Indoor swimming pool attendance and respiratory and dermal health in schoolchildren—HITEA Catalonia. Respir Med. 2014; 108(7):1056–9. https://doi.org/10.1016/j.rmed.2014.04.018 PMID: 24835075
2. Carlsen KH, Anderson SD, Bjermer L, Bonini S, Brusasco V, Canonica W, et al. Exercise-induced asthma, respiratory and allergic disorders in elite athletes: epidemiology, mechanisms and diagnosis: part I of the report from the Joint Task Force of the European Respiratory Society (ERS) and the European Academy of Allergy and Clinical Immunology (EAACI) in cooperation with GA2LEN. Allergy. 2008; 63(4):387–403. Epub 2008/03/05. https://doi.org/10.1111/j.1398-9995.2008.01662.x PMID: 18315727.
3. Mountjoy M, Fitch K, Boulet LP, Bougault V, van Mechelen W, Verhagen E. Prevalence and characteristics of asthma in the aquatic disciplines. The Journal of allergy and clinical immunology. 2015; 136 (3):588–94. Epub 2015/03/31. https://doi.org/10.1016/j.jaci.2015.01.041 PMID: 25819982.
4. Belda J, Ricart S, Casan P, Giner J, Bellido-Casado J, Torrejon M, et al. Airway inflammation in the elite athlete and type of sport. British journal of sports medicine. 2008; 42(4):244–8; discussion 8–9. Epub 2007/08/23. https://doi.org/10.1136/bjsm.2007.036335 PMID: 17711871.
5. Bernard A, Nickmilder M, Dumont X. Chlorinated pool attendance, airway epithelium defects and the risks of allergic diseases in adolescents: Interrelationships revealed by circulating biomarkers. Environmental research. 2015; 140:119–26. Epub 2015/04/12. https://doi.org/10.1016/j.envres.2015.03.034 PMID: 25863185.
6. Couto M, Stang J, Horta L, Stensrud T, Severo M, Mowinckel P, et al. Phenotyping athlete's asthma. European Respiratory Journal. 2013; 42(Suppl 57).

7. Couto M, Moreira A. The athlete "out of breath". European annals of allergy and clinical immunology. 2016; 48(2):36–45. Epub 2016/03/05. PMID: 26934737.
8. Couto M, Silva D, Santos P, Queiros S, Delgado L, Moreira A. Exploratory study comparing dysautonomia between asthmatic and non-asthmatic elite swimmers. Revista portuguesa de pneumologia. 2015; 21(1):22–9. Epub 2015/04/10. https://doi.org/10.1016/j.rppnen.2014.05.004 PMID: 25854132.
9. Hellard P, Guimaraes F, Avalos M, Houel N, Hausswirth C, Toussaint JF. Modeling the association between HR variability and illness in elite swimmers. Med Sci Sports Exerc. 2011; 43(6):1063–70. Epub 2010/11/19. https://doi.org/10.1249/MSS.0b013e318204de1c PMID: 21085039; PubMed Central PMCID: PMCPMC3309025.
10. Stang J, Stensrud T, Mowinckel P, Carlsen KH. Parasympathetic Activity and Bronchial Hyperresponsiveness in Athletes. Med Sci Sports Exerc. 2016; 48(11):2100–7. Epub 2016/10/19. https://doi.org/10.1249/MSS.0000000000001008 PMID: 27285494.
11. Canning BJ, Fischer A. Neural regulation of airway smooth muscle tone. Respiration physiology. 2001; 125(1–2):113–27. Epub 2001/03/10. PMID: 11240156.
12. Bernard A, Carbonnelle S, Dumont X, Nickmilder M. Infant swimming practice, pulmonary epithelium integrity, and the risk of allergic and respiratory diseases later in childhood. Pediatrics. 2007; 119 (6):1095–103. https://doi.org/10.1542/peds.2006-3333 PMID: 17545376
13. Bernard A, Carbonnelle S, Michel O, Higuet S, De Burbure C, Buchet JP, et al. Lung hyperpermeability and asthma prevalence in schoolchildren: unexpected associations with the attendance at indoor chlorinated swimming pools. Occup Environ Med. 2003; 60(6):385–94. https://doi.org/10.1136/oem.60.6.385 PMID: 12771389
14. Jacobs JH, Spaan S, van Rooy GB, Meliefste C, Zaat VA, Rooyackers JM, et al. Exposure to trichloramine and respiratory symptoms in indoor swimming pool workers. Eur Respir J. 2007; 29(4):690–8. Epub 2006/11/17. https://doi.org/10.1183/09031936.00024706 PMID: 17107995.
15. Andersson M, Hedman L, Nordberg G, Forsberg B, Eriksson K, Ronmark E. Swimming pool attendance is related to asthma among atopic school children: a population-based study. Environmental health a global access science source. 2015; 14:37. Epub 2015/04/19. https://doi.org/10.1186/s12940-015-0023-x PMID: 25890001; PubMed Central PMCID: PMCPMC4411937.
16. Bernard A, Nickmilder M, Voisin C, Sardella A. Impact of chlorinated swimming pool attendance on the respiratory health of adolescents. Pediatrics. 2009; 124(4):1110–8. Epub 2009/09/16. https://doi.org/10.1542/peds.2009-0032 PMID: 19752078.
17. Voisin C, Sardella A, Marcucci F, Bernard A. Infant swimming in chlorinated pools and the risks of bronchiolitis, asthma and allergy. Eur Respir J. 2010; 36(1):41–7. Epub 2010/01/16. https://doi.org/10.1183/09031936.00118009 PMID: 20075053.
18. Uyan ZS, Carraro S, Piacentini G, Baraldi E. Swimming pool, respiratory health, and childhood asthma: should we change our beliefs? Pediatric pulmonology. 2009; 44(1):31–7. Epub 2008/12/09. https://doi.org/10.1002/ppul.20947 PMID: 19061232.
19. Akopian AN, Fanick ER, Brooks EG. TRP channels and traffic-related environmental pollution-induced pulmonary disease. Seminars in immunopathology. 2016; 38(3):331–8. Epub 2016/02/04. https://doi.org/10.1007/s00281-016-0554-4 PMID: 26837756; PubMed Central PMCID: PMCPMC4896490.
20. Burbank AJ, Sood AK, Kesic MJ, Peden DB, Hernandez ML. Environmental determinants of allergy and asthma in early life. The Journal of allergy and clinical immunology. 2017; 140(1):1–12. Epub 2017/07/05. https://doi.org/10.1016/j.jaci.2017.05.010 PMID: 28673399.
21. Geppetti P, Patacchini R, Nassini R. Transient receptor potential channels and occupational exposure. Current opinion in allergy and clinical immunology. 2014; 14(2):77–83. Epub 2014/01/24. https://doi.org/10.1097/ACI.0000000000000040 PMID: 24451914.
22. Colsoul B, Nilius B, Vennekens R. On the putative role of transient receptor potential cation channels in asthma. Clin Exp Allergy. 2009; 39:1456–66. https://doi.org/10.1111/j.1365-2222.2009.03315.x PMID: 19624522
23. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J. 2005; 26(2):319–38. https://doi.org/10.1183/09031936.05.00034805 PMID: 16055882
24. Dweik RA, Boggs PB, Erzurum SC, Irvin CG, Leigh MW, Lundberg JO, et al. An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications. Am J Respir Crit Care Med. 2011; 184(5):602–15. Epub 2011/09/03. https://doi.org/10.1164/rccm.9120-11ST PMID: 21885636; PubMed Central PMCID: PMCPMC4408724.
25. Stang J, Couto M, Stensrud T, Mowinckel P, Moreira A, Carlsen KH. Assessment of Parasympathetic Activity in Athletes: Comparing Two Different Methods. Med Sci Sports Exerc. 2016; 48(2):316–22. https://doi.org/10.1249/MSS.0000000000000769 PMID: 26355246

26. Muppidi S, Adams-Huet B, Tajzoy E, Scribner M, Blazek P, Spaeth EB, et al. Dynamic pupillometry as an autonomic testing tool. Clinical autonomic research official journal of the Clinical Autonomic Research Society. 2013; 23(6):297–303. Epub 2013/07/25. https://doi.org/10.1007/s10286-013-0209-7 PMID: 23880969.

27. GINA. Global stategy for asthma management and prevention 2015. Available from: http://www.ginasthma.org.

28. Williams HC, Burney PG, Pembroke AC, Hay RJ. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. III. Independent hospital validation. The British journal of dermatology. 1994; 131 (3):406–16. Epub 1994/09/01. PMID: 7918017.

29. Voisin C, Sardella A, Bernard A. Risks of new-onset allergic sensitization and airway inflammation after early age swimming in chlorinated pools. Int J Hyg Environ Health. 2014; 217(1):38–45. Epub 2013/04/23. https://doi.org/10.1016/j.ijheh.2013.03.004 PMID: 23601779.

30. Yoo YJ, Yang HK, Hwang JM. Efficacy of digital pupillometry for diagnosis of Horner syndrome. PloS one. 2017; 12(6):e0178361. Epub 2017/06/03. https://doi.org/10.1371/journal.pone.0178361 PMID: 28575101; PubMed Central PMCID: PMCPMC5456040.

31. Silva D, Moreira A. The role of sports and exercise in allergic disease: drawbacks and benefits. Expert review of clinical immunology. 2015; 11(9):993–1003. Epub 2015/06/23. https://doi.org/10.1586/1744666X.2015.1058158 PMID: 26099040.

32. Banner K, Igney F, Poll C. TRP channels: emerging targets for respiratory disease. Pharmacol Ther. 2011; 130(3):371–84. https://doi.org/10.1016/j.pharmthera.2011.03.005 PMID: 21420429

33. Ramalho R, Pirraco A, Soares R, Palmares C, Delgado L, Moreira A. Neurogenic inflammation in the airways of elite swimmers. The Journal of sports medicine and physical fitness. 2014; 54(2):252–3. Epub 2014/02/11. PMID: 24509999.

34. Giacco SRD, Firinu D, Bjermer L, Carlsen K-H. Exercise and asthma: an overview. European Clinical Respiratory Journal. 2015; 2: https://doi.org/10.3402/ecrj.v2.27984 PubMed PMID: PMC4653278. PMID: 26672959

35. Sarmento AdO, Santos AdC, Trombetta IC, Dantas MM, Oliveira Marques AC, do Nascimento LS, et al. Regular physical exercise improves cardiac autonomic and muscle vasodilatory responses to isometric exercise in healthy elderly. Clinical Interventions in Aging. 2017; 12:1021–8. https://doi.org/10.2147/CIA.S120876 PubMed PMID: PMC5500489. PMID: 28721030

36. Lomax M. Airway dysfunction in elite swimmers: prevalence, impact, and challenges. Open Access Journal of Sports Medicine. 2016; 7:55–63. https://doi.org/10.2147/OAJSM.S88339 PubMed PMID: PMC4869851. PMID: 27274324

37. Anderson SD, Kippelen P. Exercise-induced bronchoconstriction: pathogenesis. Current allergy and asthma reports. 2005; 5(2):116–22. Epub 2005/02/03. PMID: 15683611.

38. Williams AE. Immunology: Mucosal and Body Surface Defences. UK: Wiley-Blackwell; 2012.

39. Bougault V, Turmel J, St-Laurent J, Bertrand M, Boulet LP. Asthma, airway inflammation and epithelial damage in swimmers and cold-air athletes. Eur Respir J. 2009; 33(4):740–6. Epub 2009/01/09. https://doi.org/10.1183/09031936.00117708 PMID: 19129276.

40. Font-Ribera L, Kogevinas M, Zock JP, Nieuwenhuijsen MJ, Heederik D, Villanueva CM. Swimming pool attendance and risk of asthma and allergic symptoms in children. Eur Respir J. 2009; 34(6):1304–10. https://doi.org/10.1183/09031936.00180608 PMID: 19443529

41. Font-Ribera L, Villanueva CM, Nieuwenhuijsen MJ, Zock JP, Kogevinas M, Henderson J. Swimming pool attendance, asthma, allergies, and lung function in the Avon Longitudinal Study of Parents and Children cohort. Am J Respir Crit Care Med. 2011; 183(5):582–8. https://doi.org/10.1164/rccm.201005-0761OC PMID: 20889905

42. Goodman M, Hays S. Asthma and swimming: a meta-analysis. J Asthma. 2008; 45(8):639–47. https://doi.org/10.1080/02770900802165980 PMID: 18951254

43. Jacobs JH, Fuertes E, Krop EJ, Spithoven J, Tromp P, Heederik DJ. Swimming pool attendance and respiratory symptoms and allergies among Dutch children. Occup Environ Med. 2012; 69(11):823–30. https://doi.org/10.1136/oemed-2011-100621 PMID: 22864284

44. Doherty M, Dimitriou L. Comparison of lung volume in Greek swimmers, land based athletes, and sedentary controls using allometric scaling. British journal of sports medicine. 1997; 31(4):337–41. PubMed PMID: PMC1332573. PMID: 9429014

45. Lazovic-Popovic B, Zlatkovic-Svenda M, Durmic T, Djelic M, Djordjevic Saranovic S, Zugic V. Superior lung capacity in swimmers: Some questions, more answers! Revista Portuguesa de Pneumologia (English Edition). 2016; 22(3):151–6. http://dx.doi.org/10.1016/j.rppnen.2015.11.003

46. Courteix D, Obert P, Lecoq A-M, Guenon P, Koch G. Effect of intensive swimming training on lung volumes, airway resistances and on the maximal expiratory flow-volume relationship in prepubertal girls.

European Journal of Applied Physiology and Occupational Physiology. 1997; 76(3):264–9. https://doi.org/10.1007/s004210050246 PMID: 9286607

47. Schwartz J, Litonjua A, Suh H, Verrier M, Zanobetti A, Syring M, et al. Traffic related pollution and heart rate variability in a panel of elderly subjects. Thorax. 2005; 60(6):455–61. https://doi.org/10.1136/thx.2004.024836 PubMed PMID: PMC1747419. PMID: 15923244

48. Baja ES, Schwartz JD, Coull BA, Wellenius GA, Vokonas PS, Suh HH. Structural equation modeling of parasympathetic and sympathetic response to traffic air pollution in a repeated measures study. Environmental Health. 2013; 12:81–. https://doi.org/10.1186/1476-069X-12-81 PubMed PMID: PMC3907044. PMID: 24059437

Study III

J. Cavaleiro Rufo, J. Madureira, E. Oliveira Fernandes, A. Moreira.

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REVIEW ARTICLE

Volatile organic compounds in asthma diagnosis: a systematic review and meta-analysis

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Abstract

We aimed to assess the value and classification rate of exhaled volatile organic compounds (VOCs) in asthma diagnosis. A PRISMA-oriented systematic search for published studies regarding exhaled VOCs in asthma diagnosis was conducted based on predefined criteria. Studies presenting sensitivity and specificity values for the test were included in the meta-analysis. Pooled diagnosis odds ratios (DOR), area under the curve (AUC) and positive and negative likelihood ratios (LR) for exhaled VOC profiles were calculated; and publication bias, threshold effect and heterogeneity were estimated. Eighteen studies were selected for the qualitative analysis and six met the criteria for inclusion in the quantitative analysis. Mean (95% CI) pooled DOR, positive and negative LR were 49.3 (15.9–153.3), 5.86 (3.07–11.21) and 0.16 (0.10–0.26), respectively. The AUC value was 0.94. Only three of the 18 reviewed studies performed an external validation of the model using a different data set. The results from the revised studies suggest that exhaled VOCs are promising biomarkers for asthma diagnosis and that several compounds, mainly alkanes, may be significantly associated with asthma inflammation. However, there are still various constraints associated with standardization and externally validated studies are needed to introduce exhaled VOC profiling in a clinical scenario.

The currently used clinical approaches for asthma diagnosis have multiple downsides, either considering specificity, sensitivity, invasiveness or expensiveness (1). Moreover, certain age groups, such as young children or the elderly, have a higher probability to suffer from distinct phenotypes of asthma with different metabolomics and immune responses, as well as different responses to pharmacological treatment (2–4). Concomitant respiratory diseases are very common in the aforementioned age groups and their symptoms may origin extra confounding factors for the diagnosis of asthma, such as transient virus-induced symptoms in children or chronic obstructive pulmonary disease in the elderly (5, 6). Therefore, new methodologies for an early and improved diagnosis of asthma are constantly being researched. One of these promising and noninvasive methodologies is the measurement of volatile organic compounds (VOCs) in the exhaled breath (7, 8).

To comprehend the potential of exhaled VOCs as biomarkers of asthma, it is important to understand their origin and how they relate to the pathology. Chronic airway inflammation and oxidative stress are normally associated with asthma (9). In general, the oxidative stress is caused by reactive oxygen species (ROS) produced by inflammatory cell activation resulting from the inflammation process. These ROS will promote the degradation of polyunsaturated fatty acids present in lipidic structures, such as the bronchial epithelium cell membrane,

Abbreviations
ATS, American thoracic society; COPD, Chronic obstructive pulmonary disease; DOR, Diagnostic odds ratio; eNose, Electronic nose; ERS, European respiratory society; GC-DMS, Gas chromatography coupled with a differential mobility spectrometer sensor; GC-FID, Gas chromatography coupled with flame ionization detector; GC-MS, Gas chromatography coupled with mass spectrometry; GINA, Global Initiative for Asthma; ISAAC, International Study of Asthma and Allergies in Childhood; NLR, Negative likelihood ratio; PCA, Principal component analysis; PLR, Positive likelihood ratio; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; QUADAS, Quality Assessment of Diagnostic Accuracy Studies; ROC, Receiver operating characteristics; ROS, Reactive oxygen species; SROC, Summary receiver operator characteristic; VOC, Volatile organic compound.

originating the formation of volatile hydrocarbons. Either formed by physiological or by pathophysiological events, these VOCs enter the bloodstream and are subsequently excreted in the exhaled breath in different configurations according to their origin (10–12). These configurations may also include VOCs that are locally released in the airways due to bronchial epithelial cell inflammation, forming a mixture with those produced by systemic metabolism.

Volatile organic compounds can be measured by several techniques such as gas chromatography coupled with mass spectrometry (GC-MS) or the electronic nose (eNose) technology. While GC-MS is a largely recognized and validated methodology for measuring VOCs, the efficiency of the eNose technologies in certain applications is still under discussion by the scientific community. The eNose often consists in an array of sensors that respond electronically when exposed to a mixture of volatile compounds, leading to the identification of VOC patterns or profiles, also known as breathprints or smell prints (12–16). Although Röck et al. (13) described more than 50 eNose models, the Cyranose® 320 (Sensigent, Baldwin Park, California CA, USA) is one of the currently most used models in the collection of exhaled VOCs, having showed good within-day and between-day reproducibility.

As collecting breath is a simple, inexpensive and noninvasive process, this methodology for profiling airway diseases looks particularly promising. However, one should have in mind that VOCs in exhaled breath may also be originated from exogenous sources, such as the ambient air pollution, and may enter the organism via skin, ingestion or inhalation, being also excreted in the exhaled breath (17). Therefore, it should be necessary to assess the composition and levels of VOCs in the indoor/outdoor environment where the patient spends more time of exposure in order to discriminate whether the exhaled VOCs are from endogenous or exogenous sources. In addition, the metabolism of resident or pathogenic bacteria may be a potential source of VOCs (18). Therefore, determining the specific origin of these compounds in exhaled breath may prove to be a challenge.

The clinical uses of exhaled VOCs as biomarkers for pulmonary diseases have already been properly reviewed by van de Kant et al. (12). However, presumably due to the wide range of pulmonary diseases included in the analysis, further information regarding asthma diagnosis was left to be reviewed. Moreover, several additional studies have been published concerning the clinical use of VOCs for asthma diagnosis. In this review, all the currently published methodologies regarding VOC measurement and/or profiling for the diagnosis of asthma and its phenotypes were systematically analysed and the sensitivity and specificity data retrieved from those studies were included in a meta-analysis whenever possible.

Methods

Search strategy, inclusion criteria and data extraction

A PRISMA-oriented systematic search (19) was performed until 31 October 2014 in PubMed, Scopus, Compendex, Inspec, ScienceDirect, Academic Search Complete, Web of Science and the Cochrane library. The search was conducted through the combination of the keywords 'asthma', 'exhaled', and 'VOCs' or 'volatile organic compounds'. Published peer-reviewed full-text articles in English concerning clinical studies of asthma diagnosis through VOC monitoring were assessed for eligibility. The inclusion criteria for qualitative synthesis were as follows: (a) asthma defined by a trained physician or according to official guidelines, such as those validated by the Global Initiative for Asthma or the American Thoracic Society/European Respiratory Society; (b) VOCs measured in exhaled breath; and (c) clinical studies.

The exclusion criteria consisted in (a) less than two defined groups for comparing VOC levels or VOC profiles; (b) studies focused on diagnosing symptoms of asthma, not the disease itself. Moreover, presenting the sensitivity and specificity values for the asthma diagnosis test was an additional inclusion criterion for meta-analysis. Two reviewers independently applied the inclusion criteria, and any differences were resolved by consensus.

Information regarding study design, settings, population, methodologies (including sample collection, VOC analysis techniques, targeted biomarkers, environmental air VOC assessment) and outcomes was gathered. The diagnosis tests' sensitivity and specificity data were retrieved whenever possible for meta-analysis.

Quality assessment

The quality assessment of the selected studies was conducted according to the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) tool (20). To better represent the quality assessment, QUADAS quality scores were defined: items classified as 'Yes' added 1 point to the score; items classified as 'No' and as 'Unclear' added 0 points to the score.

Statistical analysis

The statistical analysis was performed using the Stata IC software (version 13, StataCorp LP, College Station, TX, USA) and the Meta-DiSc software (version 1.4) (21). The sensitivity and specificity of the included studies were used to construct a contingency table. The bivariate meta-analysis model was employed to obtain the pooled value of sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (DOR) and generate the bivariate summary receiver operator characteristic (SROC) curve. Heterogeneity between studies was evaluated using the chi-square and I^2 tests. A value of $P < 0.05$ was considered as being statistically significant and $I^2 \geq 50\%$ indicated the existence of significant heterogeneity (22, 23). To detect the threshold effect, the Spearman's test of correlation was used to evaluate the relationship between sensitivity and specificity. The publication bias of the selected studies was assessed using Begg's funnel plot and Egger's test (24).

Results

Study selection, characterization and quality assessment

A total of 288 studies were found using the aforementioned search methodology. With the removal of duplicates, 248 studies were accepted for screening. This number was later increased to 256 after the inclusion of studies found by reference list searching. During the screening, 236 studies were excluded for not fulfilling the inclusion requirements; thus, 20 studies were selected for full revision. Two studies were later excluded according to the exclusion criteria: one study was focused on asthma symptoms diagnosis (25) and the other did not comprise two defined groups for comparisons (26). Therefore, 18 studies were included in the qualitative synthesis (27–44). However, of these 18 studies, only six presented the required data for the quantitative synthesis (Fig. 1).

Data from the 18 studies concerning asthma diagnosis by exhaled breath VOC assessment were reviewed thoroughly. In total, 560 patients with asthma diagnosed by a physician and/or according to official guidelines (including ATS, ERS, GINA and ISAAC) were investigated in seven different countries. The most recurrent methodology for sampling collection consisted in Tedlar® bags for trapping the exhaled breath (45) although the sampled volume varied between 2 ml and 10 l. The most used technique for analysing VOCs was gas chromatography coupled with mass spectrometry (GC-MS), which was used in 10 studies, followed by the Cyranose® 320 technology (five studies), whereas only three studies used gas chromatography coupled with flame ionization detector (GC-FID). One study performed exhaled breath analysis with gas chromatography coupled with differential mobility spectrometer and a mass spectrometer (GC-DMS-MS). Only three of the 18 reviewed studies performed an external validation of the model using a different data set, while the majority of the studies only used a cross-validation approach due to population size limitations, which does not provide as much certainty of the findings as external validation does. While Table 1 shows a summarized overview of the collected information, the most important outcomes are presented in the discussion. Moreover, the 18 studies were scored according to QUADAS (Table S1).

Data analysis

Six of the 18 selected studies allowed data extraction to be included in the meta-analysis (28, 29, 35, 37, 42, 44). Sensitivity and specificity of exhaled VOC profiles in asthma diagnosis tests were retrieved from these studies and grouped according to the methodology used. For the purpose of the meta-analysis, the methodology of analysis used (eNose or GC-MS) was not discriminated because it only represents different means to produce similar outcomes for diagnosing asthma (VOC profiles). The data retrieved from the aforementioned studies are presented in Table 2.

The sensitivity and specificity values of the exhaled VOC profile test in asthma diagnosis were analysed. Heterogeneity in specificity was observed among the six studies ($I^2 = 65.1\%$; $P = 0.014$), which did not occur with sensitivity ($I^2 = 46.6\%$; $P = 0.095$) (Fig. 2). Therefore, the random-effects model was employed. The pooled results reported a mean (95% CI) sensitivity of 87% (82% to 91%) and specificity of 86% (80% to 90%) (Fig. 2). The mean (95% CI) pooled PLR was 5.86 (3.07–11.21), indicating that individuals with asthma had approximately six times higher chance of presenting asthma-associated exhaled VOC profiles when compared with individuals without asthma, and the mean (95% CI) pooled NLR was 0.16 (0.10–0.26). The mean (95% CI) pooled DOR was 49.3 (15.9–153.3), and the area under the SROC curve (AUC) was 0.94 (Figs 2 and S1, respectively).

Publication bias

To assess publication bias, the Begg's funnel plot and the Egger's test were used (Figure S2). Both the funnel plot and the Egger's test P-value ($P = 0.100$) suggested no publication bias, although this observation is limited by the reduced number of studies included in the meta-analysis.

Diagnostic threshold and heterogeneity

To evaluate the diagnostic threshold, the Spearman's correlation coefficient between sensitivity and 1-specificity was calculated. The correlation coefficient was -0.429 ($P = 0.397$), suggesting that there was no heterogeneity from the threshold effect. Moreover, heterogeneity was also explored by meta-regression to assess the contribution by sample size, type of methodology used and the country where the study was performed. However, evidence of heterogeneity was not found for none of the aforementioned characteristics ($P > 0.05$).

Discussion

Our systematic review and meta-analysis showed: first, exhaled VOC profiles have high sensitivity and specificity values, respectively, 87% and 86%, for diagnosing asthma; secondly, individuals with asthma had six times higher chance of being diagnosed through exhaled VOC profiles than healthy controls; and thirdly, diagnosis odds ratios and area under the curve values are, respectively, 49.3 and 0.94. Taken together, these observations suggest that exhaled VOC profile analysis may be an accurate test for asthma diagnosis. Nevertheless, these results should be interpreted with caution because the sensitivity and specificity values were retrieved from studies using not only different analysis methods but also different algorithms for VOC profiling. Therefore, despite how much promising the meta-analysis results may seem to be, there is still much to be performed before introducing VOC biomarkers in a real clinical context.

In common with all meta-analyses, this systematic review may have included studies in which the characteristics of the subjects were too dissimilar for comparison or the spectrum of patients used was not completely representative of the one that would receive the test in practice. Nevertheless, this was

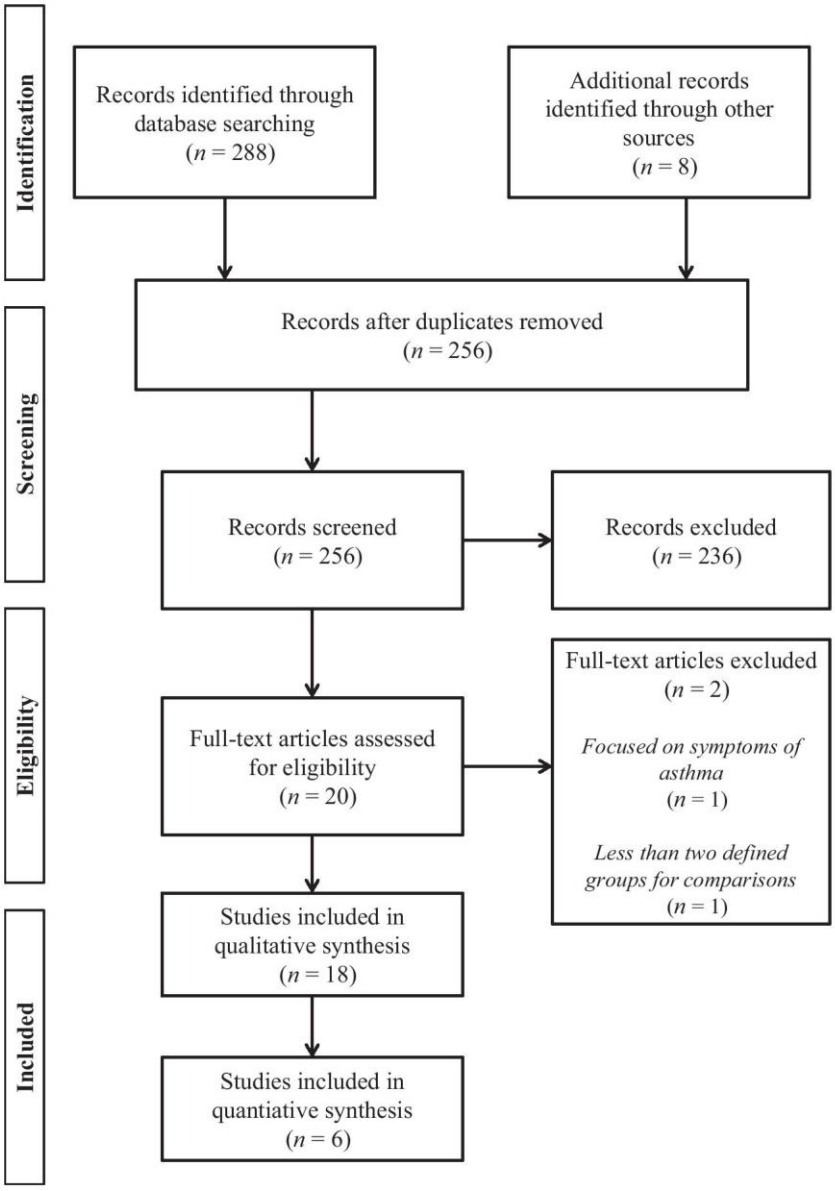

Figure 1 Summary of the literature search based on the PRISMA flow-chart (19).

considered and reported on the quality rating of the included studies, and apparently, no publication bias existed. Secondly, included studies used different methodologies for assessing VOCs (mostly Cyranose® 320 and GC-MS) and most importantly used distinct VOC profiles for diagnosing individuals with asthma, making it impossible to further investigate how the diagnostic potential correlates with these characteristics due to the reduced number of trials available. Nevertheless, as shown by the overall collected information, exhaled VOC assessment appears to be a tool with significant accuracy for asthma diagnosis. Studies with larger populations may help to better understand and validate the correct accuracy, sensitivity and specificity of exhaled VOC profiles in asthma diagnosis.

These findings are particularly important when compared with the sensitivity and specificity values of the currently used clinical tools in asthma diagnosis. For instance, the pooled sensitivity values of VOC profiles (87%) are significantly higher when compared with those obtained by spirometry considering both $FEV_1 < 80\%$ and <90% (29% and 35%, respectively) (46, 47). Bronchodilator reversibility >12% also showed a poor sensitivity value (36%) when compared with VOC profiles (48). On the other hand, the GINA self-reported symptom questionnaires showed highly skewed sensitivity/specificity ratios (10.9 for ≥1 reported symptoms and 0.2 for ≥5 reported symptoms), while VOC profiles presented a ratio approximated to 1.0 (49). Table S2 shows the sensitivity and specificity values of other asthma diagnostic tools (46).

Table 1 Characteristics of included studies on exhaled VOCs for asthma diagnosis

References	Year	Country	Objective	Participants	Breath collection	Technique	Targeted markers	Short conclusions
(38)	1997	USA	Asthma diagnosis; asthma severity monitoring	12 subjects with acute asthma, 11 subjects with stable asthma and 17 healthy controls	Tedlar bag (50 ml sampled)	GC-FID	1 VOC (Pentane)	Exhaled pentane levels are increased in patients with acute asthma and decreased significantly once acute asthma subsides
(39)	2000	United Kingdom	Asthma diagnosis; asthma severity monitoring	26 subjects with asthma and 14 healthy controls	Tedlar bag (2 ml sampled)	GC-FID	1 VOC (Ethane)	Exhaled ethane is elevated in asthma when compared to steroid-treated patients and controls
(30)	2003	USA	Asthma severity monitoring	21 subjects with asthma	Evacuated stainless steel canister	GC-MS	8 VOCs	No significant associations have been found. Benzene was the most promising marker
(36)	2007	Sweden	Asthma diagnosis	13 subjects with asthma and 14 healthy controls	Tedlar bag (3 l sampled)	GC-FID	Ethane, pentane and isoprene	There might be a minor contribution of ethane from the airways in subjects with asthma. The concentrations of isoprene are low in subjects with asthma for unknown reasons
(31)	2007	Netherlands	Asthma diagnosis; asthma severity monitoring	10 subjects with severe asthma, 10 subjects with mild asthma and 20 controls	Tedlar bag (1 l sampled)	Cyranose® 320, GC-MS	VOC profile	Smell prints of patients with mild asthma were fully separated from young controls, and patients with severe asthma could be distinguished from controls. Patients with mild and severe asthma could be less well discriminated
(23)	2009	Netherlands	Differential diagnosis (asthma *vs* COPD)	30 subjects with COPD, 20 subjects with asthma and 40 controls	Tedlar bag (10 l sampled)	Cyranose® 320	VOC profile	VOC profiling of exhaled air can distinguish patients with asthma from those with COPD or control subjects
(29)	2010	Netherlands	Asthma diagnosis	63 children with asthma and 57 healthy controls	Tedlar bag (5 l sampled)	GC-ToF-MS	VOC profile	A total of eight components discriminated with high accuracy between asthmatic and healthy children
(37)	2010	Italy	Asthma diagnosis	27 subjects with asthma and 24 controls	Tedlar bag (2 l sampled)	Metalloporphyrins-coated QMB sensor, GC-MS	VOC profile	VOC breathprints could discriminate between patients with asthma and healthy subjects with a high diagnostic performance

Table 1 (continued)

References	Year	Country	Objective	Participants	Breath collection	Technique	Targeted markers	Short conclusions
(27)	2011	Portugal	Asthma diagnosis	35 children with asthma and 15 healthy controls	Tedlar bag (1 l sampled)	GCxGC-ToF-MS	44 VOCs	Discrimination between allergic asthma and control children was attained with a high classification rate mainly by the compounds linked to oxidative stress, such as alkanes and aldehydes
(32)	2011	Netherlands	Differential diagnosis (asthma *vs* COPD)	40 subjects with COPD and 60 subjects with asthma	Tedlar bag (10 l sampled)	Cyranose® 320	VOC profile	External validity of breathprints significantly distinguishing fixed asthma from COPD and classic asthma. Discriminative accuracy was not confounded by current smoking
(35)	2011	United Kingdom	Asthma diagnosis	35 subjects with asthma and 23 healthy controls	3 l collected directly in adsorbent pipes (Tenax)	GC-MS	VOC profile	A model derived from 15 VOCs classified patients with asthma with high accuracy
(28)	2012	Portugal	Asthma diagnosis	32 children with allergic asthma and 27 healthy controls	Tedlar bag (1 l sampled)	GCxGC-ToF-MS	VOC profile	A pattern of six compounds belonging to the alkanes characterized the asthmatic population
(43)	2012	Australia	Differential diagnosis (asthma with GORD *vs* asthma without GORD)	17 subjects with COPD, 20 subjects with asthma and seven healthy controls	Tedlar bag (no volume specified)	Cyranose® 320	VOC profile	The Cyranose® 320 distinguished exhaled breath profiles of asthmatic patients with GORD from asthmatics without GORD
(34)	2013	United Kingdom	Asthma diagnosis	11 subjects with asthma and 12 healthy controls	2.5 l collected directly in adsorbent pipes (Tenax)	GC-MS	VOC profile	A panel of eight candidate markers were found to differentiate between the asthmatic and healthy children in the test cohort
(40)	2013	Netherlands	Asthma severity monitoring (prospective study)	40 children with asthma	Tedlar bag (5 l sampled)	GC-ToF-MS	VOC profile	With support vector machine analysis, the most optimal model of intravariable baseline measurements *vs* exacerbation was based on six VOCs. The model of intervariable baseline values consisted on seven VOCs
(41)	2013	USA	Asthma diagnosis; differential diagnosis (asthma *vs* COPD)	13 subjects with asthma, five subjects with COPD and 13 healthy controls	Exhaled breath condensate collector (10–15 min)	GC-DMS-MS	VOC profile	VOC profiles distinguished asthma from healthy controls. It was not possible to distinguish between COPD and asthma

Table 1 (continued)

References	Year	Country	Objective	Participants	Breath collection	Technique	Targeted markers	Short conclusions
(44)	2013	Australia	Asthma diagnosis	25 subjects with asthma and 20 controls	Inert bag (not specified)	Cyranose® 320	VOC profile	The Cyranose® 320 significantly discriminated between asthma and controls and may be used to predict the patient's response to steroids
(42)	2014	Netherlands	Asthma diagnosis (randomized trial)	76 children with asthma, 121 children with transient wheezing and 50 controls	Tedlar bag (1 l sampled)	GC-ToF-MS	VOC profile	A set of 17 VOCs discriminated preschool asthmatic children from transient wheezing children

GORD, gastro-oesophageal reflux disease; GC-MS, gas chromatography coupled with mass spectrometry; GC-FID, gas chromatography coupled with flame ionization detector; GC-DMS, gas chromatography coupled with a differential mobility spectrometer and a mass spectrometer. GC-ToF-MS, Gas chromatography coupled with time-of-flight mass spectrometry.

Exhaled VOCs for asthma diagnosis

The first reported attempt to use exhaled VOCs for asthma diagnosis was performed by Olopade et al. (38), who measured the pentane levels in the exhaled breath of 12 subjects with acute asthma, 11 with stable asthma and 17 healthy controls. Although pentane levels were shown to be significantly higher in more severe forms of asthma (see next section), they were not significantly different between individuals with stable asthma and healthy controls, suggesting that this VOC may not be adequate for the diagnosis of the disease itself. Similarly, Lärstad et al. (36) found no significant differences in pentane levels between patients with asthma and healthy controls.

Paredi et al. (39) had more success with exhaled breath ethane assessment, showing that individuals with asthma presented significantly higher levels of ethane in their breath when compared to healthy controls ($P < 0.05$). However, similarly to pentane (38), exhaled ethane was more associated as a biomarker of severity being able to significantly distinguish between steroid-naïve and steroid-treated patients with asthma ($P < 0.01$) (39). These results were later supported by Lärstad et al. (36) who showed that asthma pathophysiology may be slightly associated with exhaled ethane. For unknown reasons, Lärstad et al. (36) also found that individuals with asthma presented significantly decreased concentrations of isoprene in exhaled breath when compared to healthy controls. These results suggest that isoprene may be negatively associated with asthma. Nevertheless, as the concentrations of exhaled isoprene may be highly affected by cholesterol synthesis, diet, exercise and age, its potential as a biomarker of asthma may be limited (50).

In a clinical study comprising a population of 20 individuals with asthma and 20 controls, Dragonieri et al. (31) analysed exhaled VOC profiles with both Cyranose® 320 and GC-MS techniques. The smell prints (VOC profiles detected by electronic nose) of the patients with asthma were fully separated from healthy controls showing a significant diagnosis potential. However, explorative GC-MS analysis showed the presence of similar VOCs in asthma when compared with the control group, suggesting that further studies are required to determine specific discriminative VOC concentrations between individuals with and without the disease. Dallinga et al. (29) also analysed by GC-ToF-MS the VOC profiles in the exhaled breath collected from 63 children with asthma and 57 controls and the outcomes of the study revealed eight promising VOCs, mostly hydrocarbons, that were able to discriminate between asthmatic and healthy children achieving sensitivity of 89% and a specificity of 95%. Similar promising results were shown by Montuschi et al. (1) using a metalloporphyrins-coated QMB sensor (prototype model, University of Rome Tor Vergata), which was used to discriminate between 27 patients with asthma and 24 healthy subjects with high diagnostic performance. Classification rates were based on neural networks. Mass spectrometry fingerprinting was used in a subset of samples to confirm the eNose classification. In this case, principal component analysis (PCA) was used for classifica-

Table 2 Studies included in the meta-analysis

References	Technique	Sample size	Accuracy (%)	Sensitivity (%)	Specificity (%)
(37)	Metalloporphyrins-coated QMB sensor	12 subjects with asthma *vs* 12 controls (analysis in alveolar exhaled breath)	88	100	75
(44)	Cyranose® 320	25 subjects with asthma and 20 controls (poststeroid comparison)	82	84	80
(29)	GC-ToF-MS	63 children with asthma *vs* 57 healthy controls	92	89	95
(35)	GC-MS	35 subjects with asthma *vs* 23 healthy controls	86	85	89
(28)	GC-ToF-MS	32 children with allergic asthma *vs* 27 healthy controls	98	96	95
(42)	GC-ToF-MS	76 children with asthma *vs* 50 healthy controls	77	82	74

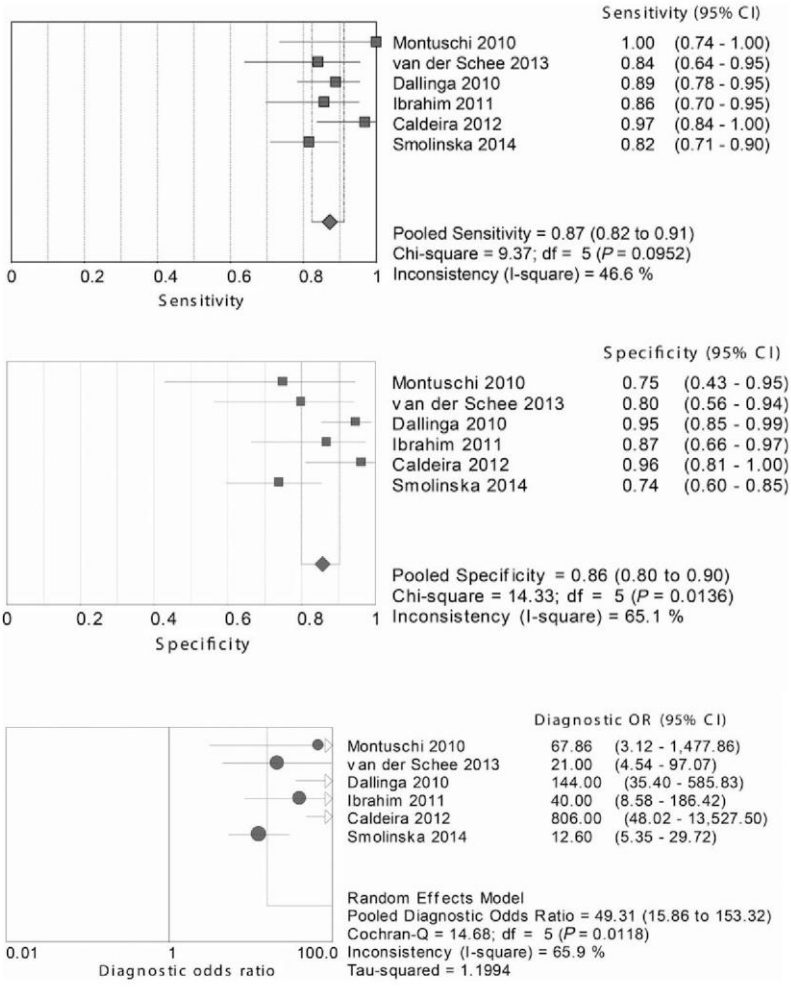

Figure 2 Forest plot of the pooled sensitivity, specificity and diagnostic odds ratio of exhaled VOC profiles in asthma diagnosis.

tion, showing clear between-group distinction. The specific compounds responsible for the discrimination were not identified. This was one of the few included studies that performed external validation of the VOC profiles used in the analysis, achieved through an alternative data set to test the classification model. Diagnostic performance of the metalloporphyrins-coated QMB sensor was also compared with FeNO and spirometry and their combination using two different procedures for breath sampling. When Caldeira et al. (27) measured 44 VOCs in the exhaled breath of 35 children with asthma and 15 healthy controls by GC-ToF-MS, they showed that it was possible to discriminate children with allergic asthma and healthy controls with 88% classification rate and that alkanes and aldehydes were the compounds

with more discriminative power. Caldeira et al. (28) did specify the most promising alkanes in a later study, which consisted on nonane, (2,2,4,6,6)-pentamethylheptane, decane, (3,6)-dimethyldecane, dodecane and tetradecane.

A VOC profile with 15 compounds obtained through GC-MS and assembled by PCA was able to differentiate between 35 individuals with asthma and 23 healthy controls, in a study performed by Ibrahim et al. (35) . Also in UK, Gahleitner et al. (34) identified through GC-MS a panel of eight compounds that were able to discriminate between 11 subjects with asthma and 12 healthy controls. However, none of these VOCs matched the promising compounds found on the aforementioned study by Caldeira et al. (28).

Using gas chromatography coupled with a differential mobility spectrometer sensor (GC-DMS) to analyse VOCs in the exhaled breath samples of 13 individuals with asthma, five subjects with COPD and 13 healthy controls, Schivo et al. (41) demonstrated that it was possible to differentiate patients with asthma from healthy individuals. However, the most discriminant compounds in this analysis could not be identified because there was no analyte diagnostic library for the GC-DMS methodology at the time of the study in spite of the parameters which could be used for species identification.

A study involving asthma diagnosis by Cyranose® 320 VOC profiling, conducted by van der Schee et al. (44), showed that this technique is able to significantly discriminate between patients with asthma and healthy controls with greater accuracy than FeNO. Unfortunately, the eNose systems are able to identify VOC breathprints but cannot specify the most discriminant compounds (15).

In a study with 252 participants comprising a random sample of children with asthma and healthy controls, Smolinska et al. (42) were able to correctly predict 80% of early asthma cases by analysing exhaled breath VOCs through GC-ToF-MS. The authors identified 14 compounds from a total of 17 that showed promising diagnosis potential. Similarly to several aforementioned studies, alkanes were the VOCs with the highest discriminant value, again suggesting that some alkanes may be linked to pathological processes associated with asthma. It is important to mention that the model used in this study was validated using an external data set.

Table 3 shows a list of the most discriminant VOCs for asthma diagnosis identified in the aforementioned studies. The results suggest that alkanes are indeed associated with asthma pathophysiology although the specific identity of the compounds appears to remain undetermined (51). Moreover, in the case of few compounds, the results appear to be contradictory in different studies. For instance, the results from Dragonieri et al. (31) suggest that acetone is positively associated with asthma, while in the study performed by Caldeira et al. (27), acetone is negatively associated with asthma. These inconsistencies may be associated with each individual intervariability, but may also be caused by exposure to different environments and, consequently, exhalation of different exogenous VOCs (52, 53). They might also reflect differences in breath sampling procedures.

Exhaled VOCs in differential diagnosis

Comorbidity is frequent in children and elderly individuals. In these circumstances, asthma diagnosis may prove to be even more difficult because several respiratory diseases share common symptoms. Such is the case of COPD, which is often misdiagnosed as asthma. Four of the 18 selected studies were focused on differentiating COPD from asthma using exhaled breath VOC analysis.

In a study conducted in the Netherlands, by Fens et al. (33), the authors aimed to correctly distinguish a population comprised by 30 subjects with COPD, 20 subjects with asthma and 40 healthy controls, analysing their exhaled breath by Cyranose® 320. The results showed that the breathprints of individuals with asthma are significantly different from those with COPD or with no respiratory diseases (controls) with an accuracy of, respectively, 96 and 95%. Although repeatable and reproducible, the results were only expressed in Cyranose® 320 breathprints; thus, no specific compounds were identified. The external validation of the mentioned results was demonstrated in a later study by Fens et al. (32) where they showed that the accuracy for discriminating asthma from COPD was not confounded by 'current smoking' status. This may be a further advantage over current diagnostic methodologies, such as FeNO assessment, that are known to be influenced by smoking (54).

Timms et al. (43) performed a study aiming to distinguish patients suffering from common obstructive lung diseases (including asthma) with concomitant gastro-oesophageal reflux disease from those without gastro-oesophageal reflux disease, using the Cyranose® 320 technology. The population included 20 individuals with asthma and the results showed that the breathprints from individuals with gastro-oesophageal reflux disease were highly distinguishable from those without reflux, in the asthma population.

In the aforementioned study performed by Schivo et al. (41), the authors hypothesized that GC-DMS could be used to differentiate between individuals with asthma and individuals with COPD, using VOC profiles. However, they observed that after analysing the VOC profiles, it was not possible to distinguish between COPD and asthma. The small size of the sampled population (13 individuals with asthma and five with COPD) was pointed by the authors as the main reason behind these results.

Despite GC-MS being the preferential technique for assessing VOCs in exhaled breath in the 18 selected studies, the Cyranose® 320 was the most used technology in differential asthma diagnosis. Consequently, no discriminant VOCs have been identified in these type of diagnosis, although the obtained results appeared to be very promising with breath prints from individuals with COPD being significantly different from those of individuals with asthma, as well as the breathprints from individuals with comorbidities, which were significantly different from those without comorbidities. However, there is not much published information concerning exhaled VOC analysis to distinguish different phenotypes of asthma (such as allergic *vs* nonallergic asthma).

Table 3 Most discriminant VOCs for asthma diagnosis identified in the revised studies

VOCs	Group	References	Identification
1,2-propanediol	Alcohol	van de Kant et al. (25)	(−)
2-butylcyclohexanol		Ibrahim et al. (35)	(−)
2-propen-1-ol		van de Kant et al. (25)	(−)
Hexadecan-2-ol		van de Kant et al. (25)	(+)
Isopropanol		Dragonieri et al. (31)	(+)
2-octenal	Aldehyde	Gahleitner et al. (34)	(+)
2-undecenal		Smolinska et al. (42)	(+)
4-methyl-2-pentenal		van de Kant et al. (25)	(+)
Decanal		Caldeira et al. (28)	(−)
Dodecanal		Caldeira et al. (28)	(−)
Nonanal		Caldeira et al. (28)	(−)
Pentadecanal		Ibrahim et al. (35)	(−)
1-(methylsulfanyl)propane	Alkane	Gahleitner et al. (34)	(+)
2,2,4-trimethylheptane		Smolinska et al. (42)	(−)
2,2-dimethylhexane		Caldeira et al. (27)	(+)
2,3,6-trimethyldecane		Caldeira et al. (27)	(+)
2,3,6-trimethyloctane		Smolinska et al. (42)	(−)
2,3-dimethyl heptane		Dragonieri et al. (31)	(+)
2,4,6-trimethyldecane		van de Kant et al. (25)	(−)
2,4-dimethylheptane*		Dragonieri et al. (31)	(+)
2,4-dimethylheptane*		Caldeira et al. (27)	(−)
2,4-dimethyloctane		Caldeira et al. (27)	(+)
2,4-dimethylheptane		Smolinska et al. (42)	(+)
2,4-dimethylpentane		Smolinska et al. (42)	(+)
2,6,10-trimethyldodecane		Smolinska et al. (42)	(−)
2,6,10-trimethyldodecane		Ibrahim et al. (35)	(+)
2,6,11-trimethyldodecane		Dragonieri et al. (31)	(+)
2,6,11-trimethyldodecane		Ibrahim et al. (35)	(+)
2-methyl-1-butene		van de Kant et al. (25)	(−)
2-methyl-decane		Ibrahim et al. (35)	(+)
2-methylhexane		Smolinska et al. (42)	(+)
2-methylpentane		Smolinska et al. (42)	(+)
3,6-dimethyldecane		Caldeira et al. (28)	(+)
3,7-dimethyl undecane		Dragonieri et al. (31)	(+)
4-methyloctane*		Dragonieri et al. (31)	(+)
4-methyloctane*		Caldeira et al. (27)	(−)
5,5-Dibutylnonane		Ibrahim et al. (35)	(−)
Alkane		Dragonieri et al. (31)	(+)
Decane		Caldeira et al. (27)	(+)
Decane		Caldeira et al. (28)	(+)
Dodecane		Caldeira et al. (27)	(+)
Dodecane		Caldeira et al. (28)	(+)
Ethane		Paredi et al. (39)	(+)
Ethane		Larstäd et al. (36)	(+)
Isododecane		Caldeira et al. (28)	(+)
Nonane		Caldeira et al. (28)	(+)
Octadecene		Gahleitner et al. (34)	(+)
Octane		Smolinska et al. (42)	(+)
Pentane		(Olopade et al. 1997)	(+)
Tetradecane		Caldeira et al. (27)	(+)
Tetradecane		Caldeira et al. (28)	(+)
Tridecane		Dallinga et al. (29)	(+)
Undecane		Dallinga et al. (29)	(−)
1-dodecene		Caldeira et al. (28)	(−)
2,4,4-trimethyl-1-pentene		van de Kant et al. (25)	(−)
2,4-octadiene		van de Kant et al. (25)	(−)

Table 3 (continued)

VOCs	Group	References	Identification
2-ethyl-1,3-butadiene		van de Kant et al. (25)	(+)
3-methyl-1-butene		van de Kant et al. (25)	(+)
4-methyl-1-decene		van de Kant et al. (25)	(+)
Benzyl alcohol	Aromatic alcohol	Ibrahim et al. (35)	(+)
Benzoic acid	Aromatic carboxylic acid	Dallinga et al. (29)	(+)
1,2,3-trimethylbenzene	Aromatic hydrocarbon	van de Kant et al. (25)	(+)
1,4-dichlorobenzene		Gahleitner et al. (34)	(+)
1,4-dichlorobenzene		van de Kant et al. (25)	(−)
1,7-dimethylnaphthalene		Gahleitner et al. (34)	(+)
1-methylethynylbenzene		van de Kant et al. (25)	(+)
2-methylnaphthalene		Smolinska et al. (42)	(−)
3-(1-methylethyl)-benzene		Dallinga et al. (29)	(+)
4-ethyl-o-xylene		Ibrahim et al. (35)	(−)
b-Cymene		Gahleitner et al. (34)	(+)
Biphenyl		Smolinska et al. (42)	(−)
Ethylbenzene		Gahleitner et al. (34)	(+)
Limonene*		Gahleitner et al. (34)	(+)
Limonene*		Smolinska et al. (42)	(−)
Naphthalene		van de Kant et al. (25)	(+)
p-xylene		Dallinga et al. (29)	(−)
Toluene		Dragonieri et al. (31)	(+)
Acetic acid	Carboxylic acid	Dragonieri et al. (31)	(+)
Butanoic acid		Dallinga et al. (29)	(+)
Pentanoic acid		van de Kant et al. (25)	(+)
1,1′:3′,1″-Ter(cyclopentane)	Cycloalkane	Dallinga et al. (29)	(+)
1-pent-2-one	Ketone	Dallinga et al. (29)	(−)
2-butanone		Ibrahim et al. (35)	(+)
6-methyl-2-heptanone		van de Kant et al. (25)	(−)
6-methyl-5-hepten-2-one		Caldeira et al. (28)	(−)
Acetone*		Dragonieri et al. (31)	(+)
Acetone*		Caldeira et al. (27)	(−)
Acetone*		Smolinska et al. (42)	(−)
Acetophenone		van de Kant et al. (25)	(−)
2,6-Di-tert-butylquinone	Miscellaneous	Ibrahim et al. (35)	(−)
3,4-Dihydroxybenzonitrile		Ibrahim et al. (35)	(+)
Allyl methyl sulphide		Ibrahim et al. (35)	(+)
Ethyl 2,2-dimethylacetoacetate		Ibrahim et al. (35)	(+)
Ethyl 4-nitrobenzoate		Ibrahim et al. (35)	(−)
Isoprene*		Larstäd et al. (36)	(−)
Isoprene*		Dragonieri et al. (31)	(+)
Isoprene*		Caldeira et al. (27)	(+)
Nitrocyclohexane		van de Kant et al. (25)	(+)
Terpinolene		Ibrahim et al. (35)	(+)

(+) indicates a positive association with asthma, whereas (−) indicates a negative association with asthma.
*Indicates conflicting outcome associations between different studies.

Exhaled VOCs in asthma severity monitoring

Several studies showed a potential to differentiate asthma severity using exhaled VOC assessment (30, 38–40, 44). This could be useful to monitor steroid treatment and to perceive exacerbations in patients with asthma.

In the aforementioned work performed by Olopade et al. (38), the results suggested that exhaled pentane levels were increased in acute asthma and dropped significantly once acute asthma subsided. Similarly, Paredi et al. (39) showed that exhaled ethane levels were also significantly increased in steroid-naïve when compared with steroid-treated patients with asthma.

Delfino et al. (30) sampled the breath of 21 individuals with asthma by GC-MS and quantified 8 VOCs including benzene, methylene chloride, styrene, tetrachloroethylene, toluene, m-, p-xylene, o-xylene and p-dichlorobenzene. The results showed no significant associations between exhaled VOC concentrations and symptoms of asthma. However, there were no alkanes on the sampled set of VOCs in this study, which supports the concept that alkanes may be the VOCs more commonly

associated with asthma pathophysiology. This may also explain the distinct results obtained in the two previously mentioned studies, by Olopade et al. (38) and Paredi et al. (39), because pentane and ethane are both alkanes.

Intra- and intersubject comparisons regarding asthma exacerbations were performed by Robroeks et al. (40) in a prospective study comprising 40 children with asthma. All samples were analysed by GC-ToF-MS. Exhaled breath was collected before and after an exacerbation in 16 children. The authors showed that, using support vector machine analysis, 6 VOCs were able to differentiate stable from exacerbated asthma measurements within patients with a classification rate of 96%. Similarly, a model derived from 7 VOCs was able to differentiate the whole exhaled breath samples of individuals with baseline measurements of stable asthma from those with baseline measurements of exacerbated asthma with a classification rate of 91%. These results suggest that not only are exhaled VOCs capable of differentiating exacerbated asthma from stable asthma measurements, they may also predict individual-specific exacerbations in children, supporting the usefulness of exhaled VOC analysis as a monitoring tool for asthma treatment.

The Cyranose® 320 technology also produced promising results in the study performed by van der Schee et al. (44). Exhaled VOC profiles were shown to predict steroid responsiveness in patients with asthma with greater accuracy than FeNO and sputum eosinophils count. These findings suggest that exhaled VOC profile analysis may be an important tool for assisting asthma treatment administration.

Exogenous VOCs in exhaled breath

When measuring exhaled VOCs as biomarkers of physiological processes, one should consider that a large part of the compounds in the exhaled breath may have exogenous origins. Potential misinterpretations may have occurred in the revised studies because the origins of certain VOCs in breath were assumed as endogenous and thus considered useful as biomarkers of asthma despite the possible contribution of food, bacteria or environmental contaminants (53, 55, 56).

The human body can absorb and exhale VOCs originated in several food products or cooking practices (57, 58). In the revised studies, the patients were usually instructed to refrain from eating or drinking in the 2 h preceding breath collection to limit the influence of exogenous VOCs on the exhaled breath. However, the kinetics of most VOCs is largely unknown and further research is needed to achieve a standardized approach for reducing the influence of food VOCs in the exhaled breath.

Eliminating the VOCs originated in the inhaled air from the breath collection requires a more challenging and complex approach because the participants cannot refrain from breathing in large periods of time preceding the breath collection. The more commonly used method for reducing the error originated by inhaled VOCs is the alveolar gradient approach. It is based on subtracting the inhaled concentration of a compound from the exhaled concentration of the same compound to determine the endogenous concentrations of each compound. If the result is positive, the compound is defined as endogenous (53, 59). Although several of the revised studies considered the alveolar gradient, there were some studies that did not perform this step. Even in the circumstances where alveolar gradient was taken into consideration, there is a possibility that other factors, such as the smoking history, may significantly hamper asthma diagnosis (17).

To avoid misinterpretations associated with exogenous VOCs in exhaled breath analysis for asthma diagnosis, all the aforementioned sources of exposure should be considered in future studies. Moreover, the standardization of proceedings to reduce the impact of exogenous VOCs in breath analysis will be an important improvement to allow reliable comparisons between studies.

Conclusions

There has been an increasing interest in noninvasive tools for asthma diagnosis. Exhaled VOCs are among the most promising biomarkers, and several compounds, mainly alkanes, have been identified as significantly associated with asthma. In this review, the more discriminant compounds identified by GC-MS in previous studies were listed and sorted as positively or negatively associated with asthma. The eNose studies also produced interesting results although it was not possible to identify the most discriminant VOCs. However, there are still various constraints associated with exhaled breath collection standardization and analysis validation. The exogenous factors that may influence exhaled VOC concentrations, such as food, bacteria and environmental contaminants, must be taken into consideration in future studies to avoid misinterpretation of the results. Moreover, pharmacological treatment must be taken into consideration because it may influence the type and quantity of VOCs in the exhaled breath. Nevertheless, if these constraints are disentangled, exhaled breath VOC profiling may be clinically used as an additional tool for asthma diagnosis or combined with other diagnostic tools, such as nuclear magnetic resonance spectroscopy of exhaled breath condensate, for a more comprehensive breathomics approach to asthma diagnosis (60).

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Authors' contribution

JCR and AM were responsible for the initial draft, systematic search and statistical analysis. JM and EOF critically revised the manuscript making important contributions to the final draft. All authors provided substantial contributions to the conception or the analysis of the work, revised the manuscript for important intellectual content, approved the final version and agreed to be accountable for all aspects of the work.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Summary receiver operating characteristics (SROC) curve for exhaled VOC profiles in asthma diagnosis.

Figure S2. Begg's funnel plot for evaluation of publication bias in the selected studies.

Table S1. Quality of studies using QUADAS (Quality assessment of studies of diagnostic accuracy included in systematic reviews).

Table S2. Sensitivity and specificity for each diagnostic test for asthma.

References

1. Montuschi P, Barnes PJ. New perspectives in pharmacological treatment of mild persistent asthma. *Drug Discovery Today* 2011;**16**:1084–1091.
2. Rufo J, Taborda-Barata L, Lourenco O. Serum biomarkers in elderly asthma. *J Asthma* 2013;**1**:1.
3. Ducharme FM, Tse SM, Chauhan B. Diagnosis, management, and prognosis of preschool wheeze. *Lancet* 2014;**383**:1593–1604.
4. Montuschi P, Malerba M, Santini G, Miravitlles M. Pharmacological treatment of chronic obstructive pulmonary disease: from evidence-based medicine to phenotyping. *Drug Discovery Today* 2014;**19**:1928–1935.
5. van der Schee MP, Hashimoto S, Schuurman AC, Repelaer van Driel JS, Adriaens N, van Amelsfoort RM et al. Altered exhaled biomarker profiles in children during and after rhinovirus-induced wheeze. *Eur Respir J* 2015;**45**:440–448.
6. de Marco R, Pesce G, Marcon A, Accordini S, Antonicelli L, Bugiani M et al. The coexistence of asthma and chronic obstructive pulmonary disease (COPD): prevalence and risk factors in young, middle-aged and elderly people from the general population. *PLoS ONE* 2013;**8**:e62985.
7. Thekedar B, Oeh U, Szymczak W, Hoeschen C, Paretzke HG. Influences of mixed expiratory sampling parameters on exhaled volatile organic compound concentrations. *J Breath Res* 2011;**5**:1752–7155.
8. Basanta M, Ibrahim B, Douce D, Morris M, Woodcock A, Fowler SJ. Methodology validation, intra-subject reproducibility and stability of exhaled volatile organic compounds. *J Breath Res* 2012;**6**:026002.
9. Jiang L, Diaz PT, Best TM, Stimpfl JN, He F, Zuo L. Molecular characterization of redox mechanisms in allergic asthma. *Ann Allergy Asthma Immunol* 2014;**113**:137–142.
10. Miekisch W, Schubert JK, Noeldge-Schomburg GFE. Diagnostic potential of breath analysis—focus on volatile organic compounds. *Clin Chim Acta* 2004;**347**:25–39.
11. Buszewski B, Kesy M, Ligor T, Amann A. Human exhaled air analytics: biomarkers of diseases. *Biomed Chromatogr* 2007;**21**:553–566.
12. van de Kant KD, van der Sande LJ, Jobsis Q, van Schayck OC, Dompeling E. Clinical use of exhaled volatile organic compounds in pulmonary diseases: a systematic review. *Respir Res* 2012;**13**:1465–9921.
13. Rock F, Barsan N, Weimar U. Electronic nose: current status and future trends. *Chem Rev* 2008;**108**:705–725.
14. Maria B, Nadia M, Marco B, Malgorzata D, Salvatore V, Maurizio S et al. Within-day and between-day repeatability of measurements with an electronic nose in patients with COPD. *J Breath Res* 2013;**7**:017103.
15. Montuschi P, Mores N, Trové A, Mondino C, Barnes PJ. The electronic nose in respiratory medicine. *Respiration* 2013;**85**: 72–84.
16. Bofan M, Mores N, Baron M, Dabrowska M, Valente S, Schmid M et al. Within-day and between-day repeatability of measurements with an electronic nose in patients with COPD. *J Breath Res* 2013;**7**:1752–7155.
17. Filipiak W, Ruzsanyi V, Mochalski P, Filipiak A, Bajtarevic A, Ager C et al. Dependence of exhaled breath composition on exogenous factors, smoking habits and exposure to air pollutants. *J Breath Res* 2012;**6**:036008.
18. Bos LDJ, Sterk PJ, Schultz MJ. Volatile metabolites of pathogens: a systematic review. *PLoS Pathog* 2013;**9**:e1003311.
19. Moher D, Liberati A, Tetzlaff J, Altman DG, The PG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med* 2009;**6**: e1000097.
20. Whiting P, Rutjes A, Reitsma J, Bossuyt P, Kleijnen J. The development of QUADAS: a tool for the quality assessment of studies of diagnostic accuracy included in systematic reviews. *BMC Med Res Methodol* 2003;**3**:25.
21. Zamora J, Abraira V, Muriel A, Khan K, Coomarasamy A. Meta-DiSc: a software for meta-analysis of test accuracy data. *BMC Med Res Methodol* 2006;**6**:31.
22. Dinnes J, Deeks J, Kirby J, Roderick P. A methodological review of how heterogeneity has been examined in systematic reviews of diagnostic test accuracy. *Health Technol Assess* 2005;**9**:1–113.
23. Higgins JPT, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ* 2003;**327**:557–560.
24. Borenstein M, Hedges LV, Higgins JPT, Rothstein HR. *Introduction to Meta-Analysis*. Oxford: Wiley, 2009.
25. van de Kant KD, van Berkel JJ, Jobsis Q, Lima Passos V, Klaassen EM, van der Sande L et al. Exhaled breath profiling in diagnosing wheezy preschool children. *Eur Respir J* 2013;**41**:183–188.
26. Reynolds JC, Jimoh MA, Guallar-Hoyas C, Creaser CS, Siddiqui S, Paul Thomas CL. Analysis of human breath samples using a modified thermal desorption: gas chromatography electrospray ionization interface. *J Breath Res* 2014;**8**:1752–7155.
27. Caldeira M, Barros AS, Bilelo MJ, Parada A, Camara JS, Rocha SM. Profiling allergic asthma volatile metabolic patterns using a headspace-solid phase microextraction/gas chromatography based methodology. *J Chromatogr A* 2011;**17**:3771–3780.
28. Caldeira M, Perestrelo R, Barros AS, Bilelo MJ, Morete A, Camara JS et al. Allergic asthma exhaled breath metabolome: a challenge for comprehensive two-dimensional gas chromatography. *J Chromatogr A* 2012;**7**:87–97.
29. Dallinga JW, Robroeks CMHHT, Van Berkel JJBN, Moonen EJC, Godschalk RWL, Jöbsis Q et al. Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children. *Clin Exp Allergy* 2010;**40**:68–76.
30. Delfino RJ, Gong H, Linn WS, Hu Y, Pellizzari ED. Respiratory symptoms and peak expiratory flow in children with asthma in relation to volatile organic compounds in exhaled breath and ambient air. *J Expo Anal Environ Epidemiol* 2003;**13**:348–363.
31. Dragonieri S, Schot R, Mertens BJ, Le Cessie S, Gauw SA, Spanevello A et al. An electronic nose in the discrimination of patients with asthma and controls. *J Allergy Clin Immunol* 2007;**120**:856–862.
32. Fens N, Roldaan AC, van der Schee MP, Boksem RJ, Zwinderman AH, Bel EH et al. External validation of exhaled breath profiling using an electronic nose in the discrimination of asthma with fixed airways obstruction and chronic obstructive pulmonary disease. *Clin Exp Allergy* 2011;**41**:1371–1378.
33. Fens N, Zwinderman AH, van der Schee MP, de Nijs SB, Dijkers E, Roldaan AC et al. Exhaled breath profiling enables discrimination of chronic obstructive pul-

monary disease and asthma. *Am J Respir Crit Care Med* 2009;**180**:1076–1082.
34. Gahleitner F, Guallar-Hoyas C, Beardsmore CS, Pandya HC, Thomas CP. Metabolomics pilot study to identify volatile organic compound markers of childhood asthma in exhaled breath. *Bioanalysis* 2013;**5**: 2239–2247.
35. Ibrahim B, Basanta M, Cadden P, Singh D, Douce D, Woodcock A et al. Non-invasive phenotyping using exhaled volatile organic compounds in asthma. *Thorax* 2011;**66**: 804–809.
36. Lärstad MAE, Torén K, Bake B, Olin AC. Determination of ethane, pentane and isoprene in exhaled air – effects of breath-holding, flow rate and purified air. *Acta Physiol* 2007;**189**:87–98.
37. Montuschi P, Santonico M, Mondino C, Pennazza G, Mantini G, Martinelli E et al. Diagnostic performance of an electronic nose, fractional exhaled nitric oxide, and lung function testing in asthma. *Chest* 2010;**137**:790–796.
38. Olopade CO, Zakkar M, Swedler WI, Rubinstein I. Exhaled pentane levels in acute asthma. *Chest* 1997;**111**:862–865.
39. Paredi P, Kharitonov SA, Barnes PJ. Elevation of exhaled ethane concentration in asthma. *Am J Respir Crit Care Med* 2000;**162**(4 Pt 1):1450–1454.
40. Robroeks CM, van Berkel JJ, Jöbsis Q, van Schooten F-J, Dallinga JW, Wouters EF et al. Exhaled volatile organic compounds predict exacerbations of childhood asthma in a 1-year prospective study. *Eur Respir J* 2013;**42**:98–106.
41. Schivo M, Seichter F, Aksenov AA, Pasamontes A, Peirano DJ, Mizaikoff B et al. A mobile instrumentation platform to distinguish airway disorders. *J Breath Res* 2013;**7**:1752–7155.
42. Smolinska A, Klaassen EMM, Dallinga JW, van de Kant KDG, Jobsis Q, Moonen EJC et al. Profiling of volatile organic compounds in exhaled breath as a strategy to find early predictive signatures of asthma in children. *PLoS ONE* 2014;**9**:e95668.
43. Timms C, Thomas PS, Yates DH. Detection of gastro-oesophageal reflux disease (GORD) in patients with obstructive lung disease using exhaled breath profiling. *J Breath Res* 2012;**6**:1752–7155.
44. van der Schee MP, Palmay R, Cowan JO, Taylor DR. Predicting steroid responsiveness in patients with asthma using exhaled breath profiling. *Clin Exp Allergy* 2013;**43**:1217–1225.
45. Beauchamp J, Herbig J, Gutmann R, Hansel A. On the use of Tedlar® bags for breath-gas sampling and analysis. *J Breath Res* 2008;**2**:046001.
46. Schneider A, Gindner L, Tilemann L, Schermer T, Dinant G-J, Meyer F et al. Diagnostic accuracy of spirometry in primary care. *BMC Pulm Med* 2009;**9**:31.
47. Smith AD, Cowan JO, Filsell S, McLachlan C, Monti-Sheehan G, Jackson P et al. Diagnosing asthma: comparisons between exhaled nitric oxide measurements and conventional tests. *Am J Respir Crit Care Med* 2004;**169**:473–478.
48. Tse SM, Gold DR, Sordillo JE, Hoffman EB, Gillman MW, Rifas-Shiman SL et al. Diagnostic accuracy of the bronchodilator response in children. *J Allergy Clin Immunol* 2013;**132**:554–559.
49. Lim SY, Jo YJ, Chun EM. The correlation between the bronchial hyperresponsiveness to methacholine and asthma like symptoms by GINA questionnaires for the diagnosis of asthma. *BMC Pulm Med* 2014;**14**:161.
50. Karl T, Prazeller P, Mayr D, Jordan A, Rieder J, Fall R et al. Human breath isoprene and its relation to blood cholesterol levels: new measurements and modeling. *J Appl Physiol* 1985;**91**:762–770.
51. Phillips M, Cataneo RN, Chaturvedi A, Danaher PJ, Devadiga A, Legendre DA et al. Effect of influenza vaccination on oxidative stress products in breath. *J Breath Res* 2010;**4**:1752–7155.
52. Weichenthal S, Kulka R, Belisle P, Joseph L, Dubeau A, Martin C et al. Personal exposure to specific volatile organic compounds and acute changes in lung function and heart rate variability among urban cyclists. *Environ Res* 2012;**118**:118–123.
53. Pleil JD, Stiegel MA, Risby TH. Clinical breath analysis: discriminating between human endogenous compounds and exogenous (environmental) chemical confounders. *J Breath Res* 2013;**7**:1752–7155.
54. Malinovschi A, Backer V, Harving H, Porsbjerg C. The value of exhaled nitric oxide to identify asthma in smoking patients with asthma-like symptoms. *Respir Med* 2012;**106**:794–801.
55. Pleil JD. Role of exhaled breath biomarkers in environmental health science. *J Toxicol Environ Health-Pt B-Crit Rev* 2008;**11**: 613–629.
56. Phillips M, Greenberg J, Awad J. Metabolic and environmental origins of volatile organic compounds in breath. *J Clin Pathol* 1994;**47**:1052–1053.
57. Kabir E, Kim KH. An investigation on hazardous and odorous pollutant emission during cooking activities. *J Hazard Mater* 2011;**188**:443–454.
58. Baranska A, Tigchelaar E, Smolinska A, Dallinga JW, Moonen EJ, Dekens JA et al. Profile of volatile organic compounds in exhaled breath changes as a result of gluten-free diet. *J Breath Res* 2013;**7**:1752–7155.
59. Phillips M, Cataneo RN, Cummin AR, Gagliardi AJ, Gleeson K, Greenberg J et al. Detection of lung cancer with volatile markers in the breath. *Chest* 2003;**123**:2115–2123.
60. Motta A, Paris D, Melck D, de Laurentiis G, Maniscalco M, Sofia M et al. Nuclear magnetic resonance-based metabolomics of exhaled breath condensate: methodological aspects. *Eur Respir J* 2012;**39**:498–500.

Study IV

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Title page

Exhaled breath condensate volatilome allows sensitive diagnosis of persistent asthma

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Abstract

Background: The diagnosis and phenotyping of paediatric asthma is particularly complex due to the lack of currently available sensitive diagnostic tools. This often results in uncertainties associated with inhaled steroid therapy prescription. Therefore, this study aimed to investigate if volatile organic compounds measured in exhaled breath condensate can be used as biomarkers for asthma diagnosis in the paediatric population.

Methods: A total of 64 participants, aged 6 to 18 years, were recruited on a random basis during visits to an outpatient allergy clinic and to a juvenile football team training session. Lung function, airway reversibility and skin prick tests were performed. Exhaled breath condensate samples were collected and breathprints were assessed using an electronic nose. Information on medical diagnosis of asthma, rhinitis and atopic dermatitis was retrieved for each participant. A hierarchical cluster model based on the volatilome profiles was then created.

Results: A two-cluster exhaled volatile organic compound-based hierarchical model was able to significantly discriminate individuals with asthma from those without the disease (AUC=0.81 [0.69 – 0.93], $p<0.001$). Individuals who had persistent asthma and were prescribed corticosteroid therapy by the physician were also significantly distinguished in the model (AUC=0.81 [0.70 – 0.92], $p<0.001$). Despite being less specific, the method showed higher overall accuracy, sensitivity and AUC values when compared to spirometry with bronchodilation.

Conclusions: Analysis of the exhaled breath condensate volatilome allowed the distinction of paediatric individuals with a medical diagnosis of asthma, identifying those in need of corticosteroid therapy.

Keywords: Asthma diagnosis; Electronic nose; Exhaled breath condensate; Multivariate analysis; Pediatric asthma; Volatile organic compounds.

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Introduction

The diagnosis and phenotyping of asthma is particularly complex in children due to concomitant virus-induced airway symptoms, transient wheezing and the difficulty in recalling history of recurrent symptoms or exacerbations, which may lead to misdiagnosis and inappropriate treatment [1-3]. In the current clinical context, and according to standardized guidelines [4,5], severe asthma is often diagnosed in children older than 6 years through long-term follow-up, consideration of the extensive differential diagnoses and by observing the child's response to bronchodilator through spirometry [6]. However, there are no specific diagnostic tools or biomarkers for detecting asthma in infancy and bronchodilator response is still one of the most frequently used measurements of reversible airflow limitation [7]. Although highly specific, absence of significant response to a bronchodilator does not preclude asthma diagnosis [6,8-10]. Therefore, a more sensitive biomarker to diagnose asthma, combining versatility, non-invasiveness and accuracy is needed.

In the last decade, exhaled volatile organic compounds (VOCs) have been increasingly used as biomarkers for asthma [11-17]. The airway inflammation characteristically present in asthma promotes the degradation of polyunsaturated fatty acids in the lipidic structures of bronchial epithelium cells membrane, promoting the formation of volatile hydrocarbons. These VOCs enter the bloodstream and are subsequently excreted in the exhaled breath in different configurations according to their origin [18-20].

Several research groups were able to build VOC models or profiles (also called *breathprints*), measured through electronic nose (eNose) or gas-chromatography coupled to mass-spectrometry (GC-MS), capable of accurately distinguishing individuals with asthma from healthy controls [11-14,21-25]. This high sensitivity method for detecting asthma inflammation might be proven useful in a real clinical context, even in childhood asthma diagnosis. Nevertheless, most of these studies were not blinded to the reference standard and missed model stability measurements, and almost all of them used exhaled breath as the VOC collection matrix. Exhaled air samples occupy large volumes, ranging from 500 mL to 10 L, and must be analysed within 6 hours of collection to avoid contamination [15,26,27]. In addition, sample bags are expensive and, although reusable, they must be cleansed with multiple nitrogen flushes [28]. All these

limitations make exhaled VOC analysis cumbersome to implement in a general clinical setting.

It is possible to measure VOCs in exhaled breath condensate (EBC), a putative good matrix for transport, storage and analysis [29]. Yet, there are no exhaled breath condensate VOC models for asthma diagnosis or severity monitoring. Therefore, the present study aimed to investigate if volatile organic compounds in exhaled breath condensate may be used as biomarkers for asthma diagnosis and/or monitoring in the paediatric population.

Materials and methods

Study design and participants

The present cross-sectional study follows the updated STARD guidelines for reporting diagnostic accuracy in studies [30]. Sample size estimation was based on previously reported accuracy measurements obtained through meta-analysis of 6 studies focused on exhaled VOCs in asthma diagnosis [26], and calculations were performed according to the classic likelihood ratio sample size estimation method for diagnostic test studies [31]. Therefore, estimating a sensitivity of 87% and a specificity of 86% for asthma diagnosis through exhaled VOC analysis, and considering a sample comparison ratio of 1.0 for balanced judgment (disease vs non-disease), power calculations showed that 42 individuals with asthma and 42 individuals without asthma would be needed to show that the index test at least surpasses the 2.90 positive likelihood ratio achieved by spirometry with bronchodilation in asthma diagnosis, according to published data [9], with a 95% confidence interval.

Participants, aged 6 to 18 years, were recruited from two distinct settings: during regular appointments to a tertiary care outpatient allergy clinic (population with a high chance of having asthma) in S. João Hospital Centre, Porto, Portugal, from May to September 2016; and during a regular training session for a local juvenile football team (population with a lower chance of having asthma) in Porto, Portugal, between 5 and 13 September 2017. Eligible participants were recruited on random basis, independently of the week day, time period of the visit, and appointed physician. No information concerning the participants medical history was obtained prior to recruitment. After obtaining the legal

guardians' informed consent and participant's agreement, airway reversibility measurements and skin prick tests (SPT) were performed, followed by EBC collection. Medical diagnosis of asthma and associated severity level (based on the administered treatment), as well as medical diagnosis of allergic rhinitis and atopic dermatitis, was established by an allergy specialist, randomly selected for each participant.

A total of 57 eligible individuals were invited to participate during the outpatient clinic visits, but only 51 were included in the study since 4 refused to partake, while the remaining 2 failed to produce valid EBC samples. During the football practice recruitments, all 13 paediatric individuals that were eligible to participate accepted the invitation. Therefore, a total of 64 participants aged 6 to 18 years were included in the study. The recruitment flow of participants is summarized in Figure 1.

The study was approved by the Ethics Committee for Health of S. João Hospital Centre (authorized at 14 April 2016) and by the National Data Protection Agency (nº 5057/2016).

Clinical assessment and reference standards

In this study, bronchodilation was performed by administering 400 µg of inhaled salbutamol, and post-bronchodilator spirometry was evaluated 20 minutes afterwards. Allergic sensitization was evaluated by SPT on the participant's forearm with extracts of *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, weed pollen mix, grass pollen mix, dog dander and *Alternaria alternata*, negative control (extracts diluent), and a positive control (histamine at 10mg/mL), all belonging to the same batches (CBF-LETI S.A., Madrid, Spain). Results were read 15 minutes afterwards. Allergic sensitization was defined by a positive SPT to at least one of the tested allergens (wheal > 3mm) coupled to a positive histamine response (wheal > 3mm) and no positivity in the negative control (wheal < 3mm) [4].

Information on medical diagnosis of asthma, rhinitis and atopic dermatitis were retrieved for each participant. The reference standard was the medical diagnosis of asthma, based on the spirometry with bronchodilation challenge results, as well as symptoms history and physical examination, according to the Global Initiative for Asthma guidelines [4]. Participants diagnosed with asthma and under maintenance inhaled steroid therapy were classified as having persistent asthma, while individuals with asthma simply under reliever inhaled therapy were regarded as having intermittent

asthma. Medical diagnosis of allergic rhinitis and atopic dermatitis was based on the allergic sensitization results as well as symptoms history and physical examination.
Asthma was diagnosed in 45 individuals with 29 being prescribed with inhaled steroid therapy (persistent asthma). Allergic rhinitis and atopic dermatitis were diagnosed in 49 and 6 individuals, respectively.

EBC collection

The Turbo 14 DECCS condenser system (Medivac, Parma, Italy) was used to collect EBC samples from included participants [32]. The device was cooled to 0 ºC prior to collection, in accordance with manufacturer's instructions. EBC samples were obtained by at least 15 minutes of normal breathing while wearing a nose clip. Generally, 800 to 1500 µL of EBC was collected from each subject being a volume of at least 600µL stipulated as the minimal requirement for a valid sample. The different sample volumes are associated with the children's tidal and minute volumes of the lungs [33].
After collection, samples were transferred to capped glass tubes and stored at -80 ºC until analysis. This procedure was performed in controlled environment through a laminar flow cabinet to decrease possible sample contamination by environmental air [34].

Electronic nose measurements

To measure breathprints in EBC samples, the Cyranose 320 (Sensigent, California, USA) electronic nose (eNose) was used. This is a handheld device capable of detecting patterns of VOCs through 32 chemical sensors based on conducting chemoresistors made from carbon black nanocomposites. The settings used for the current study are presented in Table S1.
Samples were defrosted at ambient temperature prior to processing. The whole measurement procedure was conducted in a laminar flow cabinet to prevent ambient air contamination, and the cabinet's temperature and relative humidity were continually measured. For breathprint analysis, 600 µl of EBC was transferred to 12x75mm glass assay tubes which were then covered with laboratory film. The tubes were subsequently heated in dry bath at 37 ºC for 2 minutes to increase the gas phase and an exhaust was created in the laboratory film, as previously described [29]. Afterwards, a 20g needle was attached to the Cyranose 320 snout and used to pierce the laboratory film while holding

the device above the surface. The laminar flow cabinet's air was used as reference air while baselining for 10 seconds and then a sample was drawn for another 10 seconds. This procedure was repeated for all samples.

Model construction

The 32 sensor resistance values measured by eNose were retrieved and imported as a dataframe object to the R software v3.4.2 (R Foundation, Vienna, Austria). In a first step, principal component analysis (PCA) was applied for exploratory observation of the data dimension and distribution (Figures S1). To assess clustering tendency, the Hopkin's index was calculated and a dissimilarity matrix was drawn (Figure S2). Since the Hopkin's statistic retrieved a value of 0.89, data was considered as highly clusterable [35]. Internal validation and cluster stability methods were performed and optimal scores where achieved in both cases through hierarchical clustering with k=2. To further confirm the optimal number of clusters, the total within sum of squares and the average silhouette width methods were applied. Both methods confirmed the optimal cluster score of k=2 (Figure S3 and S4). Therefore, a hierarchical model with two clusters was created using the 32 sensor resistance values, blinded to the reference standard results (Figure 2).

Statistical analysis

The SPSS® statistical package software v20.0 (IBM, New York, USA) was used for statistical analysis. The k=2 hierarchical model constructed with the 32 eNose resistance sensors was compared to the diagnostic standards. There were no missing values for any of the sensors composing the hierarchical model or individuals missing the reference standard results, in accordance with the STARD guidelines [30].

T-student test was used to find if there were differences in participants' characteristics between groups, for continuous variables. Chi-square tests were used for inferential statistics between two categorical variables and risk estimation was performed to assess the tendency of asthma severity according to each cluster of the hierarchical model. Discriminant analysis was performed to identify the most relevant variables in the

model. Receiver operating characteristic (ROC) curves were built and accuracy, sensitivity, specificity and areas under the ROC (AUC) were calculated according to standardized methods [36,37].

Results

The exhaled breath condensate VOC-based hierarchical model was able to discriminate individuals with asthma ($p<0.001$) and those with persistent disease ($p<0.001$), but not those with allergic rhinitis or atopic dermatitis (Table 1, Figure 2). Classification estimates showed a tendency to include individuals with asthma (OR=18.68; 95%CI: 4.50 to 77.24) and with a persistent disease (OR=29.50; 95%CI: 5.92 to 146.50) in cluster B (Figure 3).

Discriminant analysis showed that the highest correlation between the hierarchical model and standardized canonical discriminant functions was achieved by the persistent asthma variable (rho=0.602), followed by bronchodilation challenge positivity (rho=0.544). These results show that the analysed exhaled VOC profiles were hierarchized mainly according to the asthma status of the patients, with those prescribed with inhaled corticosteroids (persistent asthma) presenting significantly different VOC profiles from those with intermittent asthma or without asthma at all (Table 2).

Diagnostic accuracy showed AUC values of 0.81 for both asthma identification and for persistent asthma diagnosis ($p<0.001$), and achieved accuracy, sensitivity and specificity values ranging from 68.6 to 93.1%. Although specificity values were generally lower, accuracy, sensitivity and AUC parameter values obtained from exhaled breath condensate VOC analysis surpassed those from spirometry with bronchodilation in all cases (Table 3).

Discussion

The developed hierarchical model based on exhaled breath condensate VOC analysis by eNose was able to distinguish individuals with a medical diagnosis of paediatric asthma. In addition, persistent asthma patients that were under the need of inhaled corticosteroid therapy were significantly discernible with high accuracy, sensitivity and AUC values. These results show that volatilome analysis is significantly more sensitive than

spirometry with bronchodilation challenge for asthma diagnosis. Moreover, although in need of future external validation, evidence suggests a possible application of the studied methodology as a complementary diagnostic methodology to assist the physician's decision in administering corticosteroid therapy for paediatric patients with asthma. These results suggest that breathomics may be a future solution to help deliver precision therapy, answering recently proposed recommendations from an expert commission to "evolve from the use of umbrella terms to disease labels that allow for treatment guidelines to be more precise" [38].

To our knowledge, this is the first study showing a methodology cable of distinguishing individuals with asthma through eNose exhaled breath condensate analysis. Overall, it is shown that breathprints in exhaled breath condensate provide similar AUC values to those shown in other studies using gas-phased exhaled breath and/or gas-chromatography methodologies for asthma diagnosis [11-14,21,22]. In fact, the measured VOC profiles allowed the inclusion of 93.1% individuals with persistent asthma and all of those with positive bronchodilation in the same cluster of the developed model. Still, considering the high specificity of spirometry, both techniques could be used in combination to achieve a significantly reliable asthma diagnosis.

The population size in the present study may be seen as a limitation since the total required population of 84 participants to surpass the 2.90 positive likelihood ratio reported for spirometry with bronchodilation was not achieved within its 95% confidence interval. Nevertheless, despite falling slightly short on individuals without asthma, the study managed to surpass the 42 mark for minimum individuals with asthma to be included, still reaching high sensitivity values. Another limitation may be associated with not separating individuals under different doses of inhaled corticosteroid therapy, which may have generated different OR values for the actual cluster according to the extent of airway inflammation. However, since the model was built blindly to the reference standard, the number of obtainable clusters would not change.

Despite the aforementioned limitations, the present study has several important traits. Although several studies focused on asthma diagnosis through eNose technologies have already been published [26], most of these studies had either small sample sizes, biased analysis associated with the study design or statistical methods, leading to methodologies inappropriate for real clinical application. In the present survey, recruitment was performed in two different settings, an outpatient allergy clinic and a

football team training session, which hamper bias associated with the exclusive inclusion of individuals with allergic or respiratory conditions. Also, despite the fact that only football players were recruited during the training sessions, it was the variable that least influenced the model, showing that neither the recruitment setting or the participants' exercise activities have interfered with asthma diagnosis results. Stability and internal validation methods were adopted prior to model construction to assess clustering tendency. Moreover, although an exploratory analysis was performed, through PCA, the model itself was constructed blindly to the diagnostic standards (the 32 eNose sensor resistance values were the only variables used to create the model), thus reducing possible bias associated with pre-labelled group comparisons. The STARD guidelines for reporting diagnostic accuracy were followed throughout the study to avoid eventual reporting bias [30]. The reference standard results for asthma diagnosis were provided by physician's evaluation, according to standardized guidelines [4]. Finally, breathomics were performed by eNose on EBC samples, which allowed the storage and freezing of samples, a considerably inexpensive 10 second per sample analysis, and the possibility of rapidly discriminating individuals with asthma, even in a hard-to-diagnose paediatric population.

The proof of principle for loss of asthma control monitoring by eNose technology has already been showed by Brinkman and co-workers (2017) [17]. The present study's results further support this evidence, as individuals with persistent asthma were significantly distinguishable from those with intermittent asthma, which may be associated with the different degree of airway inflammation at sample collection. The odds ratio results also showed a significant tendency for medical asthma diagnosis and persistent asthma to be included in the same cluster. As expected, individuals without asthma were significantly associated with the opposite cluster of the model, thus providing a clear opposing tendency for asthma diagnosis between clusters.

Although the developed hierarchical model was already blindly built to the diagnostic standard results, an external validation of the model, tested on a new sample of individuals, should be performed to complete the STARD recommendations. Nevertheless, evidence suggests a promising future for breathomics towards a rapid and complementary asthma diagnosis and therapy administration assistance in clinical settings.

Conclusion

Our findings suggest that the analysis of exhaled breath condensate by an eNose system coupled to a post-sampling discriminant analysis may be able to distinguish paediatric individuals with asthma and to identify those in the need of inhaled maintenance steroid therapy. Moreover, the method showed higher overall accuracy, sensitivity and AUC values when compared to spirometry with bronchodilation challenge.

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References

1. Baena-Cagnani CE, Badellino HA. Diagnosis of allergy and asthma in childhood. *Current allergy and asthma reports.* 2011;11(1):71-77.
2. Contoli M, Papi A. When asthma diagnosis becomes a challenge. *The European respiratory journal.* 2010;36(2):231-233.
3. Konradsen JR, Caffrey Osvald E, Hedlin G. Update on the current methods for the diagnosis and treatment of severe childhood asthma. *Expert Rev Respir Med.* 2015;9(6):769-777.
4. Global Initiative for Asthma. *Global stategy for asthma management and prevention.* 2017.
5. Chung KF, Wenzel SE, Brozek JL, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *The European respiratory journal.* 2014;43(2):343-373.
6. Agache I, Ciobanu C. Predictive value of lung function trend and FeNO for difficult asthma in children. *Journal of investigational allergology & clinical immunology.* 2012;22(6):419-426.

7. Miller MR, Crapo R, Hankinson J, et al. General considerations for lung function testing. *The European respiratory journal.* 2005;26(1):153-161.
8. Tantisira KG, Fuhlbrigge AL, Tonascia J, et al. Bronchodilation and bronchoconstriction: predictors of future lung function in childhood asthma. *The Journal of allergy and clinical immunology.* 2006;117(6):1264-1271.
9. Schneider A, Gindner L, Tilemann L, et al. Diagnostic accuracy of spirometry in primary care. *BMC pulmonary medicine.* 2009;9:31.
10. Tse SM, Gold DR, Sordillo JE, et al. Diagnostic accuracy of the bronchodilator response in children. *The Journal of allergy and clinical immunology.* 2013;132(3):554-559.e555.
11. Caldeira M, Perestrelo R, Barros AS, et al. Allergic asthma exhaled breath metabolome: a challenge for comprehensive two-dimensional gas chromatography. *Journal of chromatography A.* 2012;1254:87-97.
12. Dallinga JW, Robroeks CM, van Berkel JJ, et al. Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2010;40(1):68-76.
13. Ibrahim B, Basanta M, Cadden P, et al. Non-invasive phenotyping using exhaled volatile organic compounds in asthma. *Thorax.* 2011;66(9):804-809.
14. Smolinska A, Klaassen EMM, Dallinga JW, et al. Profiling of Volatile Organic Compounds in Exhaled Breath As a Strategy to Find Early Predictive Signatures of Asthma in Children. *PLoS ONE.* 2014;9(4):e95668.
15. Couto M, Barbosa C, Silva D, et al. Oxidative stress in asthmatic and non-asthmatic adolescent swimmers-A breathomics approach. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology.* 2017;28(5):452-457.
16. Fitzpatrick AM, Park Y, Brown LA, Jones DP. Children with severe asthma have unique oxidative stress-associated metabolomic profiles. *The Journal of allergy and clinical immunology.* 2014;133(1):258-261.e251-258.
17. Brinkman P, van de Pol MA, Gerritsen MG, et al. Exhaled breath profiles in the monitoring of loss of control and clinical recovery in asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2017;47(9):1159-1169.

18. Buszewski B, Kesy M, Ligor T, Amann A. Human exhaled air analytics: biomarkers of diseases. *Biomed Chromatogr.* 2007;21(6):553-566.
19. van de Kant KD, van der Sande LJ, Jobsis Q, van Schayck OC, Dompeling E. Clinical use of exhaled volatile organic compounds in pulmonary diseases: a systematic review. *Respir Res.* 2012;13:117.
20. Miekisch W, Schubert JK, Noeldge-Schomburg GF. Diagnostic potential of breath analysis--focus on volatile organic compounds. *Clin Chim Acta.* 2004;347(1-2):25-39.
21. Montuschi P, Santonico M, Mondino C, et al. Diagnostic performance of an electronic nose, fractional exhaled nitric oxide, and lung function testing in asthma. *Chest.* 2010;137(4):790-796.
22. van der Schee MP, Palmay R, Cowan JO, Taylor DR. Predicting steroid responsiveness in patients with asthma using exhaled breath profiling. *Clin Exp Allergy.* 2013;43(11):1217-1225.
23. Dragonieri S, Schot R, Mertens BJ, et al. An electronic nose in the discrimination of patients with asthma and controls. *J Allergy Clin Immunol.* 2007;120(4):856-862.
24. Fens N, Roldaan AC, van der Schee MP, et al. External validation of exhaled breath profiling using an electronic nose in the discrimination of asthma with fixed airways obstruction and chronic obstructive pulmonary disease. *Clin Exp Allergy.* 2011;41(10):1371-1378.
25. Schivo M, Seichter F, Aksenov AA, et al. A mobile instrumentation platform to distinguish airway disorders. *J Breath Res.* 2013;7(1):1752-7155.
26. Rufo JC, Madureira J, Fernandes EO, Moreira A. Volatile organic compounds in asthma diagnosis: a systematic review and meta-analysis. *Allergy.* 2016;71(2):175-188.
27. Mochalski P, Wzorek B, Sliwka I, Amann A. Suitability of different polymer bags for storage of volatile sulphur compounds relevant to breath analysis. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2009;877(3):189-196.
28. Caldeira M, Barros AS, Bilelo MJ, Parada A, Camara JS, Rocha SM. Profiling allergic asthma volatile metabolic patterns using a headspace-solid phase microextraction/gas chromatography based methodology. *Journal of chromatography A.* 2011;1218(24):3771-3780.

29. Hüttmann E-M, Greulich T, Hattesohl A, et al. Comparison of Two Devices and Two Breathing Patterns for Exhaled Breath Condensate Sampling. *PLoS ONE.* 2011;6(11):e27467.
30. Bossuyt PM, Reitsma JB, Bruns DE, et al. STARD 2015: An Updated List of Essential Items for Reporting Diagnostic Accuracy Studies. *Radiology.* 2015;277(3):826-832.
31. Simel DL, Samsa GP, Matchar DB. Likelihood ratios with confidence: sample size estimation for diagnostic test studies. *Journal of clinical epidemiology.* 1991;44(8):763-770.
32. Montuschi P. Analysis of exhaled breath condensate in respiratory medicine: methodological aspects and potential clinical applications. *Therapeutic advances in respiratory disease.* 2007;1(1):5-23.
33. Liu J, Thomas PS. Relationship between exhaled breath condensate volume and measurements of lung volumes. *Respiration; international review of thoracic diseases.* 2007;74(2):142-145.
34. Yang X, Chen Q, Zeng J, Zhang JS, Shaw CY. A mass transfer model for simulating volatile organic compound emissions from 'wet' coating materials applied to absorptive substrates. *International Journal of Heat and Mass Transfer.* 2001;44(9):1803-1815.
35. Banerjee A, Dave RN. Validating clusters using the Hopkins statistic. Paper presented at: 2004 IEEE International Conference on Fuzzy Systems (IEEE Cat. No.04CH37542); 25-29 July 2004, 2004.
36. Baratloo A, Hosseini M, Negida A, El Ashal G. Part 1: Simple Definition and Calculation of Accuracy, Sensitivity and Specificity. *Emergency.* 2015;3(2):48-49.
37. Carter JV, Pan J, Rai SN, Galandiuk S. ROC-ing along: Evaluation and interpretation of receiver operating characteristic curves. *Surgery.* 2016;159(6):1638-1645.
38. Pavord ID, Beasley R, Agusti A, et al. After asthma: redefining airways diseases. *Lancet.* 2018;391(10118):350-400.

Table 1 – Differences between the hierarchical model clusters, created using the 32 sensor resistance values measured by the eNose, according to participants' characteristics.

Characteristics	Cluster A	Cluster B	*p*
n (males)	26 (17)	38 (24)	0.855
Age (years, mean ± sd)	10.7 (±3.5)	12.1 (±3.1)	0.223*
Height (cm, mean ± sd)	148.2 (±19.3)	155.2 (±15.2)	0.050*
Weight (kg, mean ± sd)	43.3 (±15.4)	51.4 (±14.9)	0.920*
Positive BD (n)	0	20	**<0.001**
Positive SPT (n)	22	29	0.418
Medical diagnosis of asthma (n)	10	35	**<0.001**
Intermittent asthma (n)	8	8	0.378
Persistent asthma (n)	2	27	**<0.001**
Allergic rhinitis (n)	21	28	0.511
Atopic dermatitis (n)	3	3	0.623

p values in bold correspond to significant differences between clusters. Differences calculated through qui-square tests, except for continuous variables (marked with *), which were calculated through t-student tests. BD: Spirometry with bronchodilation; SPT: Skin prick tests.

Table 2 – Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions. Variables are ordered by absolute size of correlation within function.

Discriminating variables	**rho***
Persistent asthma	0.602
Positive bronchodilation	0.544
Weight	0.156
Age	0.146
Height	0.134
Intermittent asthma	-0.077
Skin prick tests	-0.052
Allergic rhinitis	-0.042
Sex	0.035
Atopic dermatitis	-0.028
Recruitment setting	-0.009

*Correlation coefficient for standardized canonical discriminant functions

Table 3 – Diagnostic accuracy parameters of the developed hierarchical model based on EBC VOC analysis through eNose in comparison with spirometry with bronchodilation.

	Method	Accuracy (%)	Sensitivity (%)	Specificity (%)	AUC (CI 95%)	*p*
Asthma	BD	61.3	44.4	100	0.72 (0.60 - 0.84)	**0.005**
diagnosis	eNose	79.7	77.8	84.2	0.81 (0.69 - 0.93)	**<0.001**
Intermittent	BD	59.4	31.3	68.8	0.50 (0.34 - 0.67)	1.000
asthma	eNose	40.6	50	37.5	0.56 (0.40 - 0.73)	0.457
Persistent	BD	70.3	51.7	85.7	0.69 (0.55 - 0.82)	**0.010**
asthma	eNose	79.7	93.1	68.6	0.81 (0.70 - 0.92)	**<0.001**

BD: Spirometry with bronchodilation; eNose: Exhaled VOC model measured by electronic nose.

Figure captions (no colour for in-text figures):

Figure 1 – Flow of participants through the study according to STARD [30].

Figure 2 – Dendrogram of the hierarchical model. All presented conditions have been independently diagnosed by a physician according to standardized guidelines. It is possible to observe a tendency for more asthma cases in cluster B (left branch).

Figure 3 – Classification rates estimates (OR with 95% confidence interval) between the two clusters of the hierarchical model. Results represent the chance of participants with specific characteristics (represented in the y axis) being aggregated in a cluster of the developed hierarchical model.

Supplementary material:

Table S1 – Cyranose 320 eNose settings for EBC analysis.

Figure S1 – Spatial distribution of the eNose sensor resistance values based on two components, after exploratory principal component analysis.

Figure S2 – Dissimilarity matrix of the eNose sensor data. The agglomerated colours suggest highly clusterable data, with a Hopkin's statistic index of 0.89.

Figure S3 - Total within sum of squares (elbow) method for determining optimal number of clusters. The "elbow" of the curve indicates the suitable number of clusters for a given dataframe. In this case, the elbow bends at k=2, suggesting that data may be grouped in two different clusters.

Figure S4 – Silhouette method for determining optimal number of clusters. The peak of the curve indicates the suitable number of clusters for a given dataframe. In this case, the curve peaks at k=2, suggesting that data may be grouped in two different clusters.

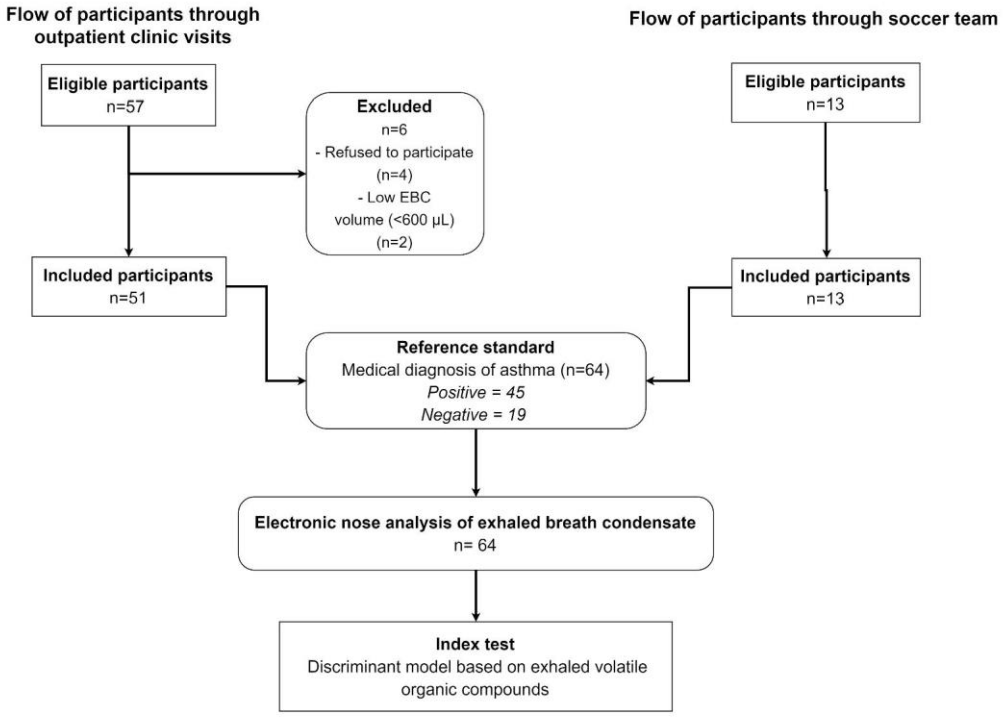

Figure 1 - Flow of participants through the study according to STARD 30.

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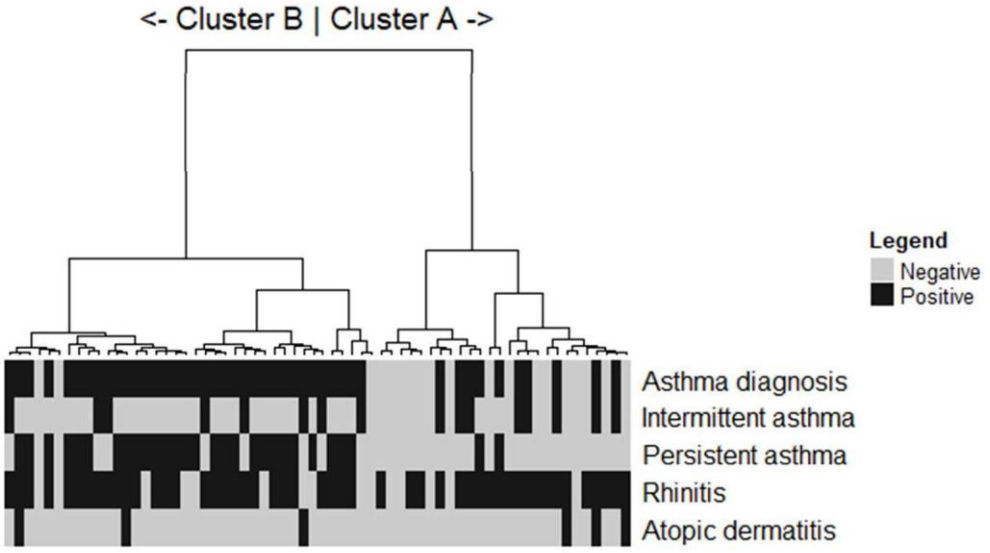


Figure 2 - Dendrogram of the hierarchical model. All presented conditions have been independently diagnosed by a physician according to standardized guidelines. It is possible to observe a tendency for more asthma cases in cluster B (left branch).

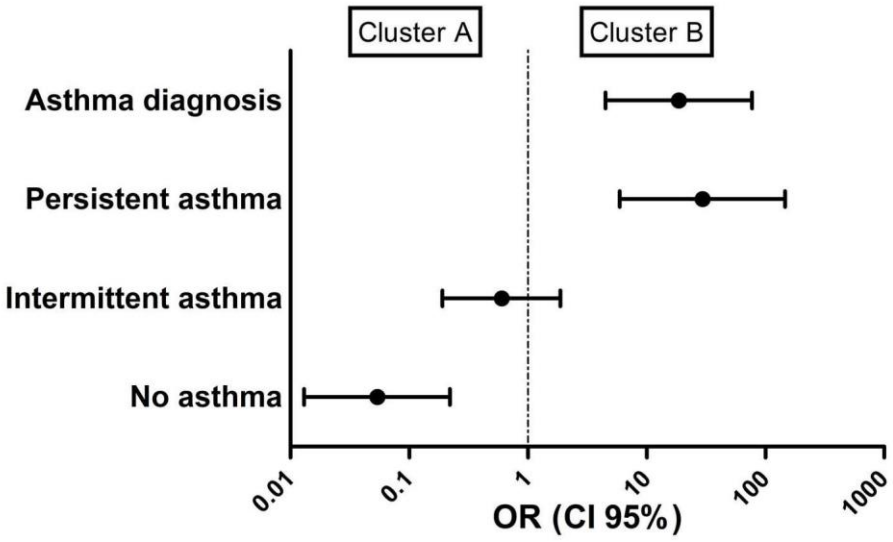


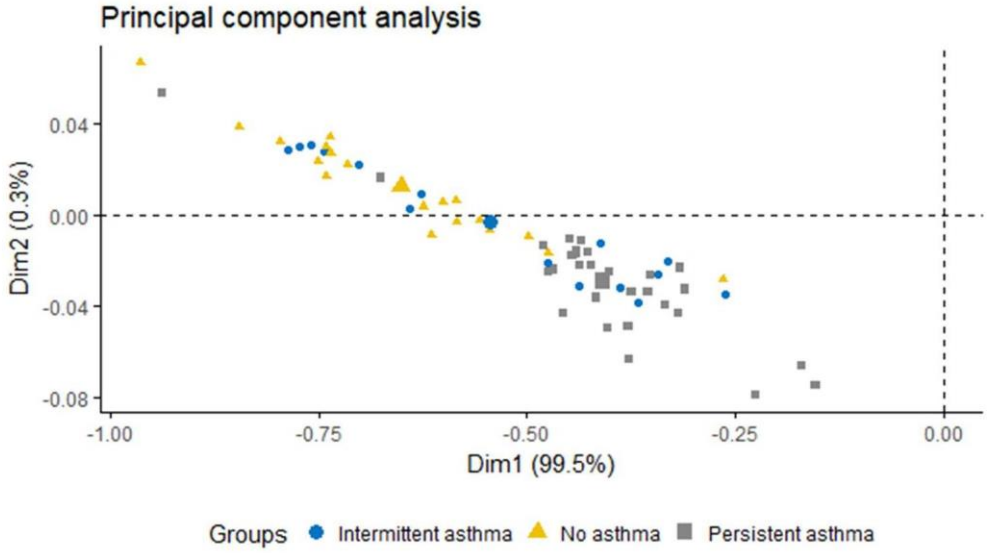
Figure 3 – Classification rates estimates (OR with 95% confidence interval) between the two clusters of the hierarchical model. Results represent the chance of participants with specific characteristics (represented in the y axis) being aggregated in a cluster of the developed hierarchical model.

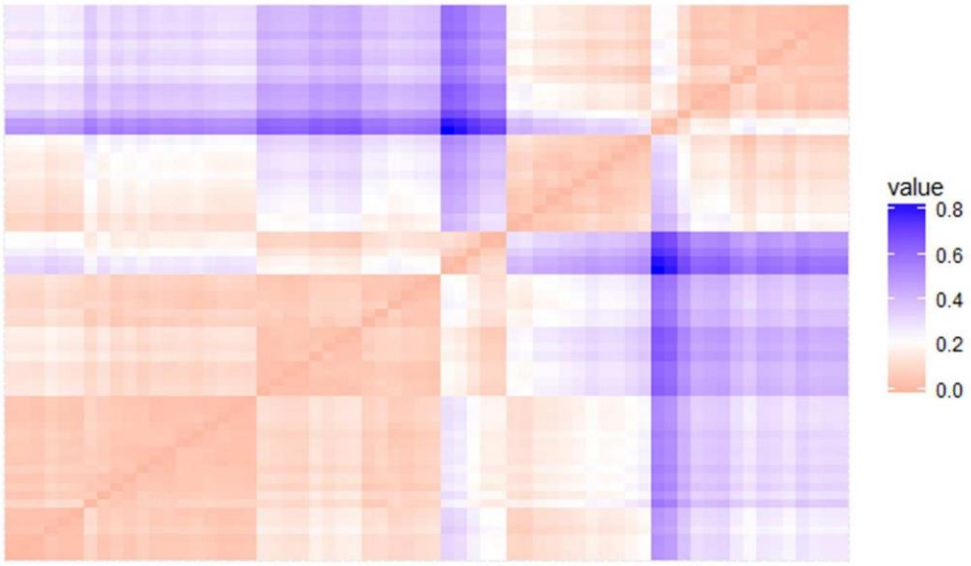
149x90mm (300 x 300 DPI)

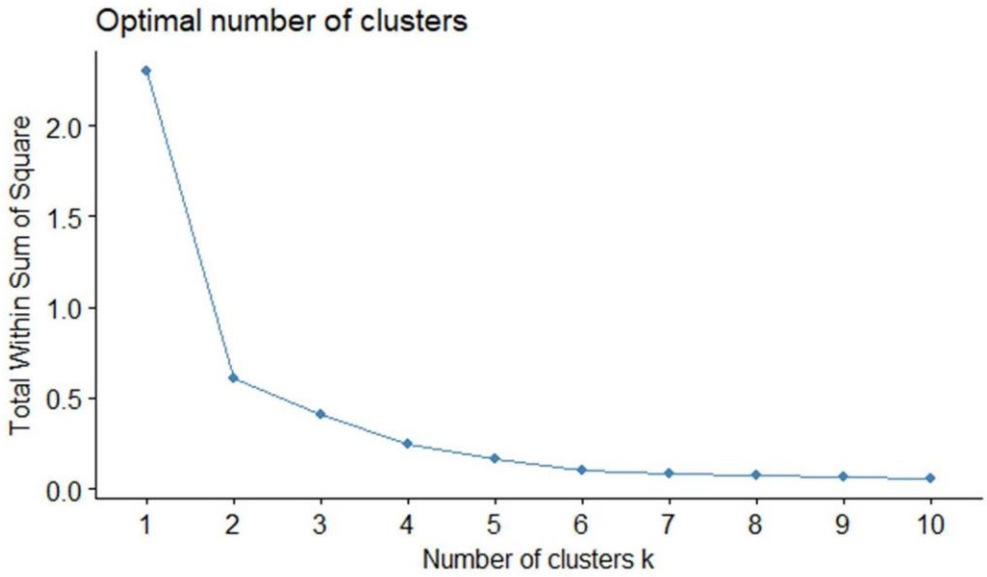
Table S1 – Cyranose 320 eNose settings for EBC analysis.

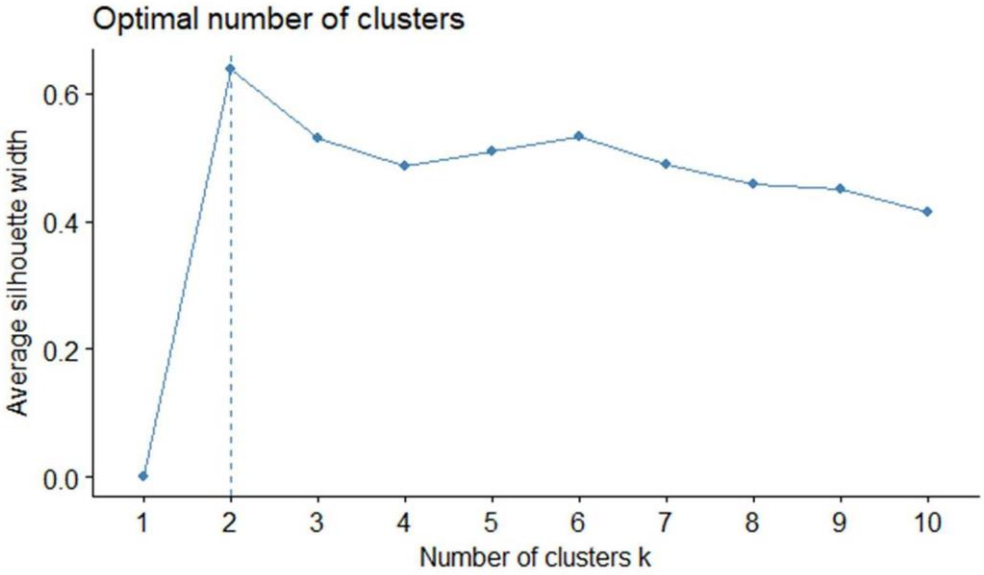
Setting	Time	Speed
Baseline purge	10s	Medium
Sample draw 1	10s	Medium
Sample draw 2	0s	*n.a.*
Snout removal	10s	*n.a.*
1st sample gas purge	20s	High
1st air intake purge	30s	High
2nd sample gas purge	20s	High
2nd air intake purge	0s	*n.a.*

n.a.: not applicable


Principal component analysis
0.04
0.00
-0.04
-0.08
Dim2 (0.3%)
-1.00
-0.75
-0.50
-0.25
0.00
Dim1 (99.5%)
Groups
Intermittent asthma
No asthma
Persistent asthma




Optimal number of clusters
Total Within Sum of Square
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
7
8
9
10
Number of clusters k


Optimal number of clusters
Average silhouette width
0.0
0.2
0.4
0.6
1
2
3
4
5
6
7
8
9
10
Number of clusters k

Attachment I

Attachment I - Quality of the systematically reviewed studies (study III).

Study	**QUADAS items**														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Quality Score
Olopade *et al*. [31]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Unclear	10
Paredi *et al*. [32]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Unclear	Yes	Yes	Unclear	9
Delfino *et al*. [23]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
Larstad *et al*. [29]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
Dragonieri *et al*. [24]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
Fens *et al*. [26]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
Dallinga *et al*. [22]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
Montuschi *et al*. [30]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Unclear	10
Caldeira *et al*. [20]	No	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Unclear	Unclear	Yes	Yes	Yes	9
Fens *et al*. [25]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
Ibrahim *et al*. [28]	No	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	10
Caldeira *et al*. [21]	No	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Unclear	Unclear	Yes	Yes	Yes	9
Timms *et al*. [36]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Unclear	Unclear	Yes	Yes	Yes	10
Gahleitner *et al*. [27]	No	No	Yes	Yes	Yes	Yes	Yes	Yes	No	Unclear	Unclear	Yes	Yes	Unclear	8
Robroeks *et al*. [33]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Schivo *et al*. [34]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
van der Schee *et al*. [37]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	12
Smolinska *et al*. [35]	No	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Unclear	Unclear	Unclear	Yes	Yes	9